REMARKS

Reconsideration and allowance are respectfully requested.

Claims 25-30, 32 and 34 are now pending, with Claim 25 being the sole independent claim.

Claims 1-24, 31 and 33 have been cancelled without prejudice to or disclaimer of the subject matter recited therein.

Turning now to the Office Action mailed April 21, 2003:

The title of the invention has been amended as suggested.

Regarding the Section 101 (utility) rejection of Claims 25-30, 32 and 34, Applicants respectfully traverse.

First, page 1, lines 17-19 of the instant specification states the following:

> "Specific sucrose carrier sequences from these crop plants should find use in controlling the timing and extent of phenomena such as grain fill duration that are important factors in crop yield and quality."

Wilhelm et al. (*Crop Sci.* 39:1733-1741 (1999)) (cited in a Supplemental IDS filed simultaneously herewith), entitled "Heat Stress during Grain Filling in Maize: Effects on Kernel Growth and Metabolism", states the following on page 1734, column 1, paragraph 3:

> "Grain-filling duration may be determined by a number of factors including sucrose availability to the kernel (Afuakwa et al., 1984) and activity levels of enzymes involved in sugar and starch metabolism in the kernel (Singletary et al., 1994). Similarly, the rate of grain filling may be affected by sucrose concentration in the kernel (Jenner, 1970) and activity levels of enzymes in the pathway of starch biosynthesis (Jenner et al., 1993; Keeling et al., 1993, 1994)."

Second, Applicants believe Lemoine (*Biochim. Biophys. Acta* 1465:246-262 (2000)) (hereinafter "Lemoine"; cited in a Supplemental IDS filed simultaneously herewith), entitled "Sucrose transporters in plants: update on function and structure", to be well-known to one of ordinary skill in the art.

Lemoine discloses a transmembrane α-helices model of a celery sucrose transporter AgSUT1 in Figure 3 (see page 255). The green circles represent residues that are conserved in all 12 sucrose transporter sequences found in Table 1 (page 249). The yellow circles correspond to residues conserved in 11 out of 12 sequences. See Figure 3 and corresponding discussion in Lemoine. Lemoine notes at page 256 that "amino acid conservation occurs in regions which could be related to transmembrane α-helices, whereas a high variability was found in the N- and C-termini and the central loop. The highest conservation is found is [sic] transmembrane segments 1, 2 and 11."

Appendix A, attached hereto, is a sequence alignment of the maize SEQ ID NO:2 of the pending claims, NCBI GI 2723471 (rice SEQ ID NO:26 of the instant

application), NCBI GI 5771354 (corn sucrose transporter) and NCBI GI 4091891 (celery sucrose transporter; disclosed in Lemoine). The conserved amino acids among all four sequences can be found in Consensus #1. The four conserved cysteine residues are indicated by boxes. SEQ ID NO:2 has 129 out of 135 conserved residues (green circles) and all four conserved cysteine residues.

Attached as Appendix B is a chart setting forth a comparison of the percent identity (and percent divergence in the lower half triangle), using the Clustal alignment method, between the four sucrose transporters found in Appendix A.

In view of the foregoing, Applicants respectfully request withdrawal of the Section 101 (utility) rejection of Claims 25-30, 32 and 34.

Regarding the Section 112, 1st paragraph (enablement) rejection of Claims 25-30, 32 and 34 on the same grounds as the Section 101 (utitlity) rejection, Applicants submit that the above remarks in response to the Section 101 rejection are applicable here.

Regarding the Section 112, 1st paragraph (enablement) rejection of Claims 25(a) and 26, Applicants respectfully traverse.

As discussed *supra*, the specification coupled with the extensive knowledge about conserved residues present in the art provides specific guidance to one of ordinary skill as to which structures are likely to have enzyme activity. The experimentation necessary to determine activity is not undue in this field, where the level of skill is very high. Applicants disclose methods for expressing the recombinant constructs in monocot, dicot and microbial cells (see prophetic Examples 4-6 of the instant specification). With the expressed polypeptide, activity may be determined using routine tests.

Regarding the Section 112, 1st paragraph (enablement) rejection of Claim 34, Applicants respectfully traverse. The instant specification discloses methods for expressing the recombinant constructs in monocot and dicot cells, and for selecting and regenerating plants therefrom (see prophetic Examples 4 and 5 of the instant application). Applicants submit that one of ordinary skill in the art could carry out these methods without undue experimentation.

In view of the foregoing, Applicants respectfully request withdrawal of all of the Section 112, 1st paragraph (enablement) rejections.

Regarding the Section 112, 1st paragraph (written description) rejection of Claim 34, Applicants respectfully traverse.

The specification at page 1, line 37 to page 2, line 5 (in the Summary of Invention section) states:

> "In a further embodiment, the instant invention concerns a transformed host cell comprising in its genome a chimeric gene encoding a sucrose transport protein, operably linked to suitable regulatory sequences. Expression of the chimeric gene

> results in production of altered levels of the encoded protein in the transformed host cell. The transformed host cell can be of eukaryotic or prokaryotic origin, and include cells derived from higher plants and microorganisms. The invention also includes transformed plants that arise from transformed host cells of higher plants....".

Furthermore, prophetic Examples 4 and 5 disclose methods for expressing recombinant DNA constructs in monocot and dicot cells, and transformed plants regenerated therefrom.

Applicants submit that these disclosures in the specification constitute a sufficient written description of Claim 34 as now recited. Furthermore, Applicants submit that one of ordinary skill in the art, upon reading these disclosures in the specification, would readily understand that Applicant was in possession of Claim 34 as now recited. Neither the Examples nor the Summary of Invention are meant to be limited to any particular SEQ ID NOs recited in the instant application; rather, one of ordinary skill would understand the Examples and Summary of Invention to encompass any of the SEQ ID NOs.

In view of the foregoing, withdrawal of the Section 112, 1st paragraph (written description) rejection of Claim 34 is respectfully requested.

Applicants believe the foregoing to be resposive to each and every point raise in the Office Action. Allowance of the above-referenced application is respectfully requested.

Please charge any fees or credit any overpayment of fees which are required in connection herewith to Deposit Account No. 04-1928 (E. I. du Pont de Nemours and Company).

Applicants' undersigned may be reached at the below-listed numbers.

Respectfully submitted,

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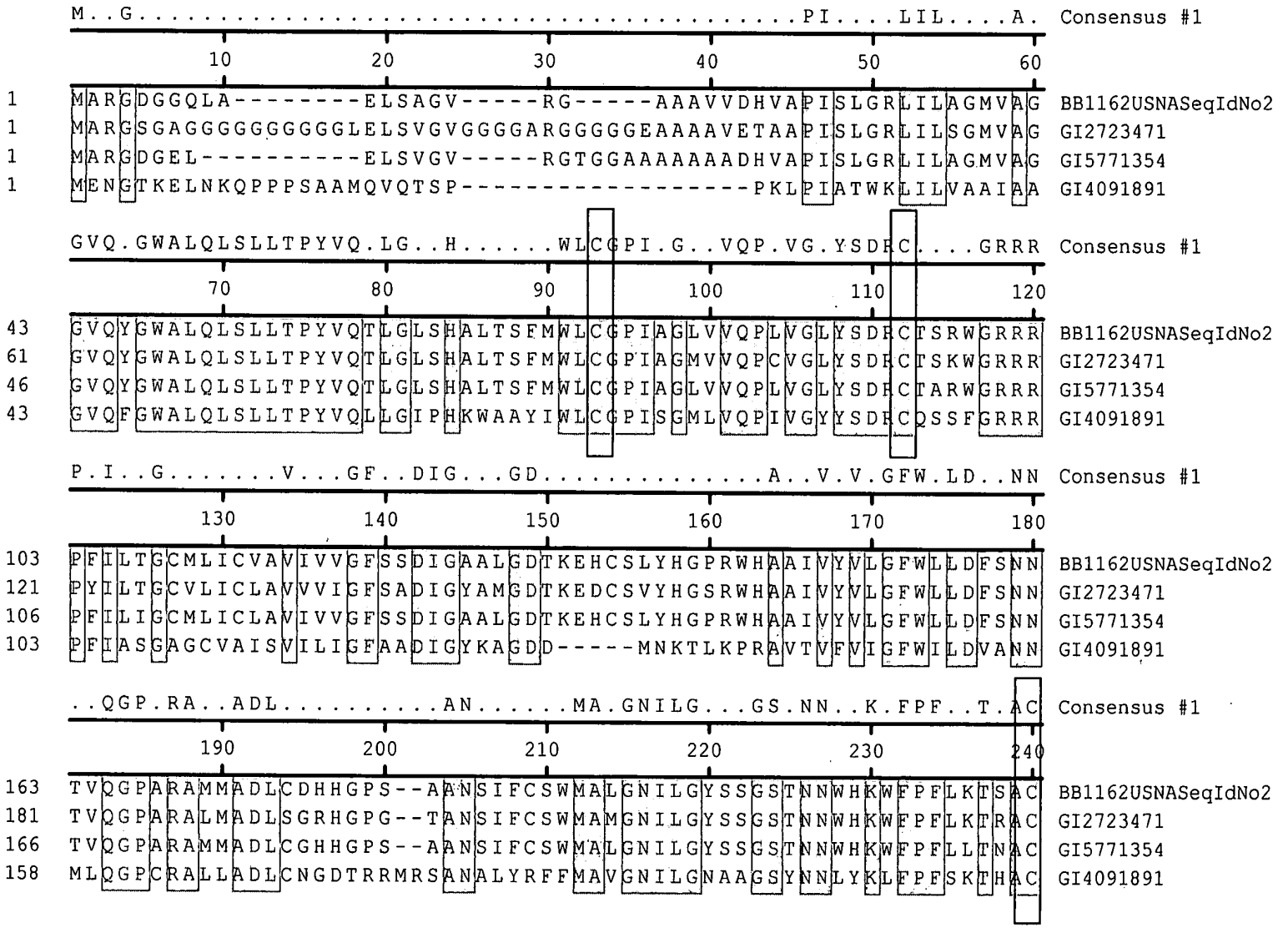
REGISTRATION NO. 41,881
TELEPHONE: 302-992-4929
FACSIMILE: 302-892-1026

Dated: 21 October 2003

Appendix A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1 MARGSGAGGGGGGGGGGLELSVGVGGGGARGGGGGEAAAAVETAAPISLGRLILSGMVAG GI2723471
1 MARGDGEL----------ELSVGV-----RGTGGAAAAAAADHVAPISLGRLILAGMVAG GI5771354
1 MENGTKELNKQPPPSAAMQVQTSP----------PKLPIATWKLILVAAIAA GI4091891
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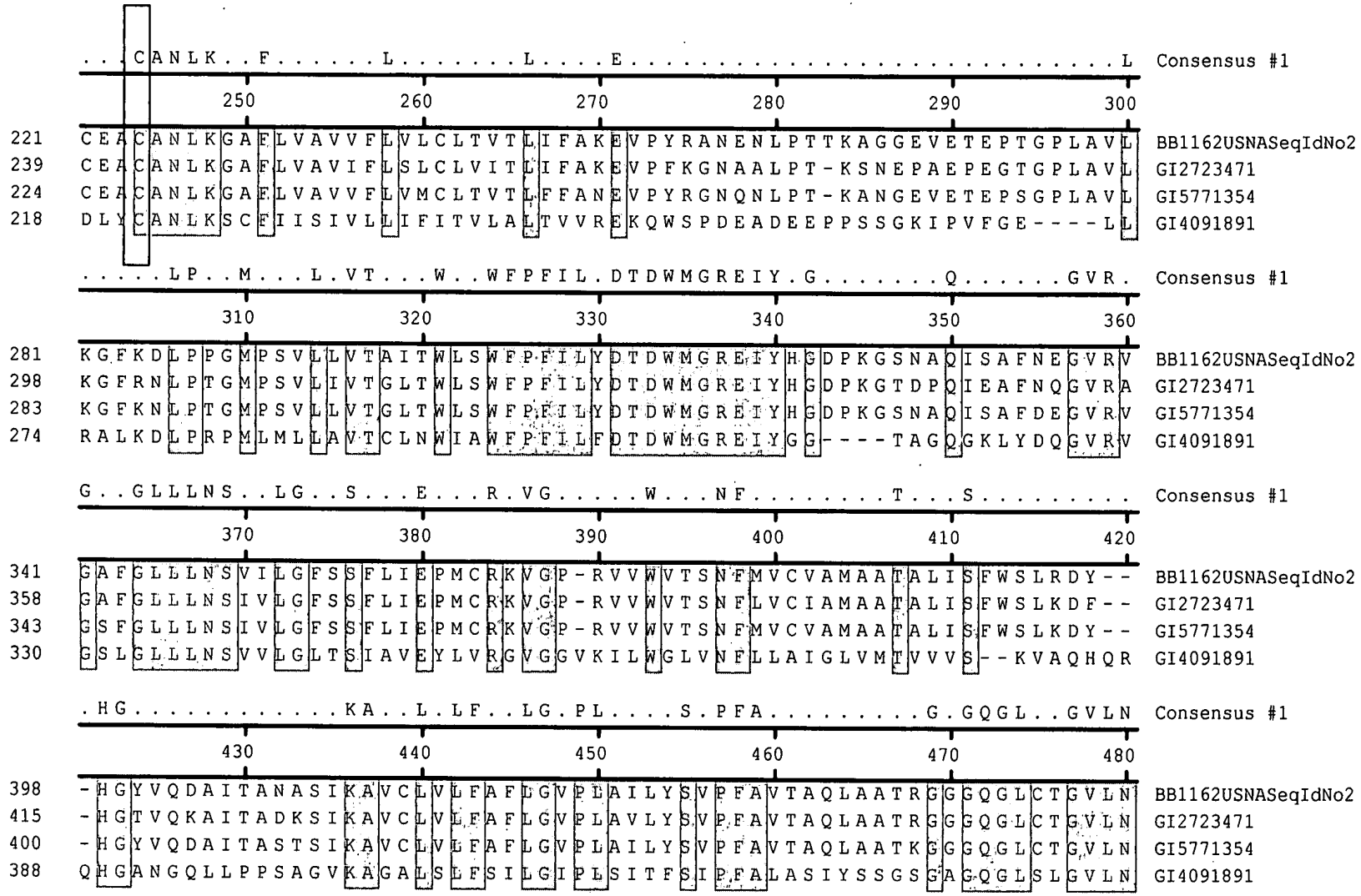
70 80 90 100 110 120
43 GVQYGWALQLSLLTPYVQTLGLSHALTSFMWLCGPIAGLVVQPLVGLYSDRCTSRWGRRR BB1162USNASeqIdNo2
61 GVQYGWALQLSLLTPYVQTLGLSHALTSFMWLCGPIAGMVVQPCVGLYSDRCTSKWGRRR GI2723471
46 GVQYGWALQLSLLTPYVQTLGLSHALTSFMWLCGPIAGLVVQPLVGLYSDRCTARWGRRR GI5771354
43 GVQFGWALQLSLLTPYVQLLGIPHKWAAYIWLCGPISGMLVQPIVGYYSDRCQSSFGRRR GI4091891
P.I..G.....V...GF..DIG...GD.....A..V.V.GFW.LD..NN Consensus #1
130 140 150 160 170 180
103 PFILTGCMLICVAVIVVGFSSDIGAALGDTKEHCSLYHGPRWHAAIVYVLGFWLLDFSNN BB1162USNASeqIdNo2
121 PYILTGCVLICLAVVVIGFSADIGYAMGDTKEDCSVYHGSRWHAAIVYVLGFWLLDFSNN GI2723471
106 PFILIGCMLICLAVIVVGFSSDIGAALGDTKEHCSLYHGPRWHAAIVYVLGFWLLDFSNN GI5771354
103 PFIASGAGCVAISVILIGFAADIGYKAGDD-----MNKTLKPRAVTVFVIGFWILDVANN GI4091891
..QGP.RA..ADL.....AN.....MA.GNILG...GS.NN..K.FPF..T.AC Consensus #1
190 200 210 220 230 240
163 TVQGPARAMMADLCDHHGPS--AANSIFCSWMALGNILGYSSGSTNNWHKWFPFLKTSAC BB1162USNASeqIdNo2
181 TVQGPARALMADLSGRHGPG--TANSIFCSWMAMGNILGYSSGSTNNWHKWFPFLKTRAC GI2723471
166 TVQGPARAMMADLCGHHGPS--AANSIFCSWMALGNILGYSSGSTNNWHKWFPFLLTNAC GI5771354
158 MLQGPCRALLADLCNGDTRRMRSANALYRFFMAVGNILGNAAGSYNNLYKLFPFSKTHAC GI4091891

Appendix A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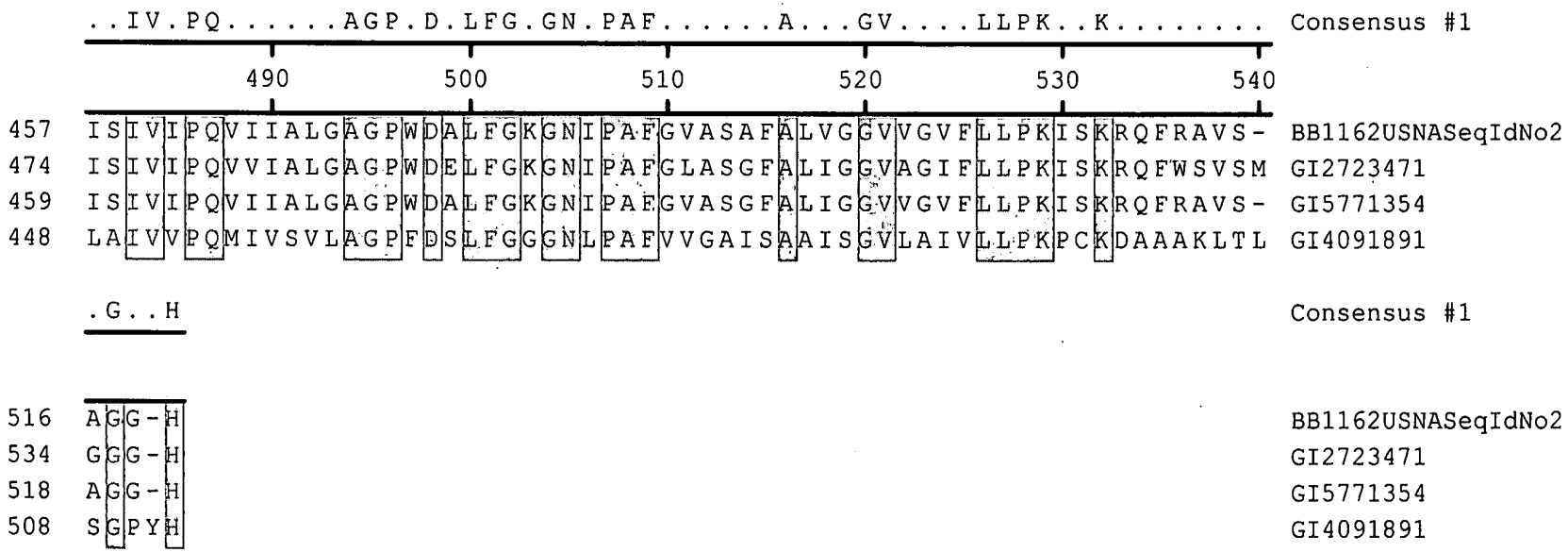
239 C E A C A N L K G A F L V A V I F L S L C L V I T L I F A K E V P F K G N A A L P T - K S N E P A E P E G T G P L A V L GI2723471
224 C E A C A N L K G A F L V A V V F L V M C L T V T L F F A N E V P Y R G N Q N L P T - K A N G E V E T E P S G P L A V L GI5771354
218 D L Y C A N L K S C F I I S I V L L I F I T V L A L T V V R E K Q W S P D E A D E E P P S S G K I P V F G E - - - - L L GI4091891
. . . . . L P . . M . . . L . V T . . . W . . W F P F I L . D T D W M G R E I Y . G . . . . . Q . . . . . G V R . Consensus #1
310 320 330 340 350 360
281 K G F K D L P P G M P S V L L V T A I T W L S W F P F I L Y D T D W M G R E I Y H G D P K G S N A Q I S A F N E G V R V BB1162USNASeqIdNo2
298 K G F R N L P T G M P S V L I V T G L T W L S W F P F I L Y D T D W M G R E I Y H G D P K G T D P Q I E A F N Q G V R A GI2723471
283 K G F K N L P T G M P S V L L V T G L T W L S W F P F I L Y D T D W M G R E I Y H G D P K G S N A Q I S A F D E G V R V GI5771354
274 R A L K D L P R P M L M L L A V T C L N W I A W F P F I L F D T D W M G R E I Y G G - - - - T A G Q G K L Y D Q G V R V GI4091891
G . . G L L L N S . . L G . . S . . . E . . . R . V G . . . . . W . . . N F . . . . . T . . . S . . . . . Consensus #1
370 380 390 400 410 420
341 G A F G L L L N S V I L G F S S F L I E P M C R K V G P - R V V W V T S N F M V C V A M A A T A L I S F W S L R D Y - - BB1162USNASeqIdNo2
358 G A F G L L L N S I V L G F S S F L I E P M C R K V G P - R V V W V T S N F L V C I A M A A T A L I S F W S L K D F - - GI2723471
343 G S F G L L L N S I V L G F S S F L I E P M C R K V G P - R V V W V T S N F M V C V A M A A T A L I S F W S L K D Y - - GI5771354
330 G S L G L L L N S V V L G L T S I A V E Y L V R G V G G V K I L W G L V N F L L A I G L V M T V V V S - - K V A Q H Q R GI4091891
. H G . . . . . K A . . L . L F . . L G . P L . . . . S . P F A . . . . . G . G Q G L . . G V L N Consensus #1
430 440 450 460 470 480
398 - H G Y V Q D A I T A N A S I K A V C L V L F A F L G V P L A I L Y S V P F A V T A Q L A A T R G G G Q G L C T G V L N BB1162USNASeqIdNo2
415 - H G T V Q K A I T A D K S I K A V C L V L F A F L G V P L A V L Y S V P F A V T A Q L A A T R G G G Q G L C T G V L N GI2723471
400 - H G Y V Q D A I T A S T S I K A V C L V L F A F L G V P L A I L Y S V P F A V T A Q L A A T K G G G Q G L C T G V L N GI5771354
388 Q H G A N G Q L L P P S A G V K A G A L S L F S I L G I P L S I T F S I P F A L A S I Y S S G S G A G Q G L S L G V L N GI4091891

Appendix A



Consensus #1: When all match the residue on the Consensus show the residue of the Consensus, otherwise show '.'

Shade (with black at 40% fill) residues that match the consensus named "Consensus #1" exactly.

Conserved cysteine residues are indicated by boxes.

Appendix B

Percent Identity

Divergence

	1	2	3	4	
1	[redacted]	81.7	91.5	39.8	1
2	17.8	[redacted]	83.7	40.2	2
3	6.5	17.0	[redacted]	39.6	3
4	95.7	96.0	93.6	[redacted]	4
	1	2	3	4	

BB1162USNASEQIDNO2 *Zea mays*

GI 2723471 (SEQ ID NO:26) *Oryza sativa*

GI 5771354 *Zea mays*

GI 4091891 *Apium graveolens*

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Review

Sucrose transporters in plants: update on function and structure

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Abstract

In plants, sucrose is the major transport form for photoassimilated carbon and is both a source of carbon skeletons and energy for plant organs unable to perform photosynthesis (sink organs). As a molecule translocated over distance, sucrose has to pass through a number of membranes. Membrane transport of sucrose has therefore been considered for a long time as a major determinant of plant productivity. After several decades of physiological and biochemical experiments measuring the activity of sucrose carriers, unequivocal evidence came from the first identification of a cDNA coding a sucrose carrier (*SoSUT1*, Riesmeier et al. (1992) EMBO J. 11, 4705–4713). At present 20 different cDNAs encoding sucrose carriers have been identified in different plant species, in both dicots and monocots (one case). The total number is increasing rapidly and most importantly, it can be guessed from the results obtained for *Arabidopsis*, that in each species, sucrose transporters represent a gene family. The sequences are highly conserved and those carriers display the typical 12 transmembrane α-helices of members of the Major Facilitator superfamily. Yeast expression of those carriers indicate that they are all influx carriers, all cotransport sucrose and proton and that their affinity for sucrose is surprisingly similar (0.2–2 mM). All their characteristics are in agreement with those demonstrated at the physiological level in plants. These characteristics are discussed in relation to the function in plants and the few data available on the structure of those transporters in relation to their function are presented.

Keywords: Plant; Membrane transport; Sucrose transporter; Sugar transporter; Structure/function

1. Introduction

Plants are autotrophic organisms that are able to synthesise complex molecules by reducing C, N and S from simple molecules. As a major translocatable product of photosynthesis, sucrose (glucose+fructose) is the main soluble component of the phloem sap [1]. Even in species translocating either derivatives of sucrose (raffinose, stachyose and verbascose) or polyols (mannitol, sorbitol), sucrose is still present in significant amount in the phloem sap. Selection of sucrose as the major transport sugar in plant has been related to its non-reducing nature and relative insensitivity to metabolism [2]. This represents an advantage for a substrate translocated over long distance in the plant [3], allowing transport without the problem of metabolism easily encountered with glucose. The β-fructoside nature of sucrose is unusual. The only other non-reducing disaccharide is trehalose, found in fungi and insect haemolymph [3].

In plants, sucrose is transported from synthesising (source) organs to sink organs where it is stored (as

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PII: S0005-2736(00)00142-5

sucrose or, e.g., as starch) or metabolised. Sucrose is therefore a source of carbon skeletons but also an energy vector. Recently, sucrose has been considered as a signal molecule involved in the regulation of gene expression by the so-called sugar-sensing pathway [4,5].

In plants, sucrose is transported over long distance in solution in the phloem sap. This flow of sap occurs in a specialised network of cells, called the sieve elements. Sieve elements lose their nucleus and many organelles during differentiation, but stay connected to companion cells, cells with a high metabolic activity. Sieve elements are connected to form sieve tubes that oppose very little resistance to the flow of sap. In most species, at least crop species, the sieve element/companion cell complex (SE-CCC) is symplasmically isolated from the surrounding cells. The high solute content of the phloem sap (sucrose, but also amino acids and ions among other compounds) and the high osmotic pressure (30 bar) of the SE-CCC compared to mesophyll cells (13 bar [6]) has led to the concept of phloem loading. According to this concept, the high osmotic pressure in the SE-CCC is due to an active 'loading' of solutes (mainly sucrose) in those cells. However, this concept may not be universal as, in some species such as willow [7], no solute concentration difference exists between the SE-CCC and the surrounding cells. The movement of the sap in the phloem occurs through mass flow [8,9], the driving force for this movement being the entry of sucrose and subsequently water in the sieve tubes in the source organ while, at the other end of the conduit in the sink organs, the continuous unloading of solutes and water would maintain the flow.

The accumulation of sucrose in the sieve tube requires the presence of a sucrose transporter to drive this active accumulation. This points to the importance of this carrier system for the translocation of solutes from source to sink organs. The existence of a carrier system specific for sucrose and responsible for the entry of sucrose in the phloem has been postulated in the late 1970s [3], the energy for this transport being the proton gradient established by a H^+/ATPase located in the plasma membrane. Although the difference in sucrose concentration between the compartments defined by the different membranes is not precisely known (due to anatomical constraints), the existence of carriers have been postulated (or clearly demonstrated) in the following membranes (Fig. 1). Beginning with the synthesis of sucrose, the first transmembrane event is the transport of sucrose in the vacuole, which determines the pool of sucrose available for export (sucrose is temporarily stored into the vacuole). Then, sucrose has to exit the mesophyll cell (step 2) and, from the apoplasm, enter the phloem cells (step 3). To exit the long distance pathway, several ways are possible as different situations are encountered among species (apoplastic vs. symplastic unloading, for a general review see [10]). When sucrose is unloaded into the apoplasmic space (step 4), it can then be taken up as sucrose into the sink cells (step 5) or cleaved by an invertase to hexoses that are transported by specific carriers [11]. Then sucrose is used for sink growth or development (metabolic sink) or can be stored as sucrose in the vacuoles of the storage cells (sugar beet, sugar cane, step 6 in Fig. 1). There might be some additional steps, such as retrieval along the

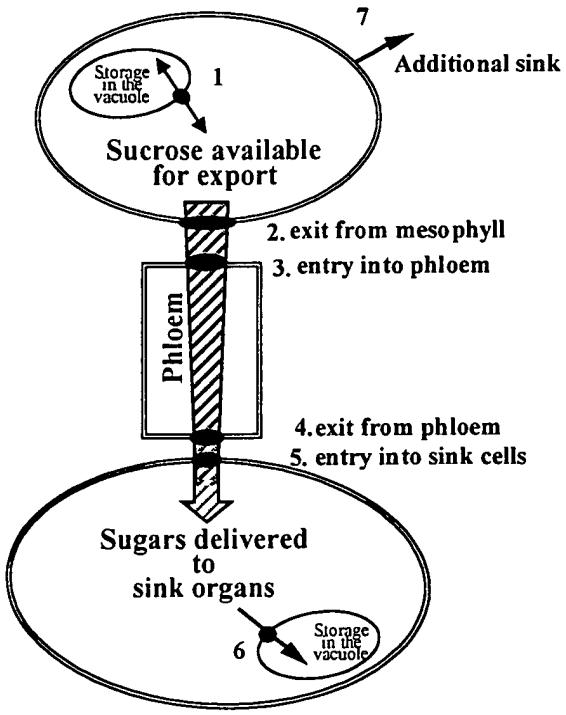

Fig. 1. Transmembrane steps mediated by a sucrose transporter. The flow of sucrose from the source organ (upper part) to the sink organs (lower part) through the phloem is represented as a large arrow, and the numbers refer to the different events of membrane transport discussed in the text.

translocation path; however, the corresponding carriers are responsible for the same type of transport as the one described in step 3. Transmembrane steps have also been involved in the transport of solutes from host plant to fungus (powdery mildew, see [12]) and are represented as step 7 in Fig. 1.

According to the different steps identified, sucrose transporters in plants can be of three types: plasma membrane influx carriers responsible for the entry of sucrose into cells that are of the proton/sucrose symporter type; tonoplast carriers have been proposed to work as sucrose/proton antiporters [13] as the vacuole is acidic compared to the cytoplasm; and finally, plasma membrane efflux carriers responsible, e.g., for the unloading of sucrose in sink organs or for sucrose exit from the mesophyll cells in close vicinity to the phloem (steps 2 and 4). Efflux carriers could, in theory, either be facilitators or antiporters.

In order to get more information on the transport of sucrose in plants, readers are referred to [14] whereas information on the physiological and biochemical characterisation of sucrose transport activities can be found in [15].

2. Identification of sucrose carriers

2.1. *Influx carriers*

The existence of specific carriers responsible for the crossing of sucrose through membranes has been postulated for many years. However, during the 1980s several groups designed strategies to identify precisely these protein(s). One strategy was based on the property of a thiol reagent NEM (*N*-ethylmaleimide) to bind irreversibly to the sucrose carrier, close enough to the binding site so that the presence of sucrose could prevent the binding of NEM. By using radiolabelled NEM for binding experiments in the presence or absence of sucrose, a group of polypeptides (molecular mass of 42 kDa) was identified in the plasma membrane of sugar beet leaves [16]. A similar method had been used before for the identification of the lactose permease from *Escherichia coli* [17]. Antibodies raised against the 42 kDa polypeptides were shown to inhibit specifically the uptake of sucrose [18,19] and the same antibodies were used to immunopurify proteins showing transport activity when reconstituted into liposomes [20]. Due to the presence of several polypeptides in the 42 kDa fraction used, the definitive identification of the sucrose carrier was not possible.

Meanwhile, a second approach based on the photolabelling of the sucrose carrier was developed. A sucrose analogue was designed (6′-deoxy-6′(4-azido-2-hydroxy)-benzamido-sucrose) and shown to competitively inhibit sucrose uptake [21]. This molecule was able to photo-affinity label a 62 kDa protein in soybean [22]. Antibodies raised against this protein showed a correlation between the appearance of a sucrose transport activity and the detection of the 62 kDa protein in different cell types [23]. The corresponding cDNA was cloned from a soybean library but showed no sign of coding a typical membrane protein. Surprisingly, when this cDNA is expressed in yeast, it confers to the yeast the ability to take up sucrose, although in a non-saturable way [24]. The role of the 62 kDa binding protein in sucrose transport (interaction with the H^+/sucrose cotransporter?) is therefore not clear today.

The third and successful trial for the identification of the sucrose carrier was based on a totally different approach. The yeast complementation system had already been used for the identification of several genes (reviewed in [25]). Therefore Riesmeier et al. [26] developed a yeast strain that could only grow on sucrose when complemented with a sucrose carrier. For this purpose they first prepared a yeast strain mutated in the secreted invertase so that sucrose could not be cleaved outside the yeast cell. Then a sucrose metabolising activity (sucrose synthase) was expressed inside the cell. When a sucrose carrier was expressed in this strain, sucrose could enter the cell, be metabolised and support growth. The yeast strain (SUSY7) was complemented with a spinach leaf cDNA library and plated on sucrose as the sole carbon source. Among the growing clones, seven were shown to contain a plasmid with an insert size of 1.95 kb. Apart from conferring the ability to take up sucrose to yeast cells, the protein coded by the cDNA showed some of the typical features of membrane carriers (high overall hydrophobicity and presence of alternating hydrophobic and hydrophilic regions, see below). The first identified sucrose carrier was then called SoSUT1 (for *Spinacia oleracea* SUcrose Transporter). The same method was then used

to identify the sucrose carrier from potato leaves called StSUT1 (*Solanum tuberosum* SUcrose Transporter [27]). All the subsequently identified carriers have been obtained by hybridisation screening or PCR amplification from these initial sequences. All the sucrose carriers cloned to date are listed in Table 1. Four sequences obtained during the sequencing of the *Arabidopsis* genome and showing high identity levels with sucrose carriers have also been included. As can be seen no common nomenclature has been approved yet! There is a majority in favour of using the original name of SUT for SUcrose Transporter, although SUC (for SUcrose Carrier) and Scr (for Sucrose CarrieR) are also found. Once the whole family of sucrose carrier is identified, it will certainly be necessary to have a common nomenclature.The nomenclature used for the four genomic sequences of *Arabidopsis* (AtSUTX1–4) is only indicative. The length of the different carriers is rather similar (around 510 amino acid residues) and this corresponds to a molecular mass of approx. 55 kDa. The longest sequence is one from *Arabidopsis* (AtSUTX4, 594 a.a.) obtained from the genome sequencing programme. The discrepancies between calculated (55 kDa) and measured molecular mass in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (45–48 kDa [28]) are due to the very hydrophobic nature of these proteins.

2.2. Vacuolar carriers

As indicated before, vacuolar carriers are supposed to work as H^+/sucrose antiporters [13]. An immunological approach by Getz et al. [29] gave some indications that the sucrose transport activity from red beet tonoplast was associated with polypeptides in the range 55–60 kDa when reconstituted in proteoliposomes. However, no further characterisation was reported. Only in one case was a protein shown to be associated with the tonoplast [30]. However, the corresponding cDNA is not closely related to all the other sucrose carriers of plant (but there is no indication that a sucrose/proton antiporter and a sucrose/proton symporter should share extensive sequence homologies) and no function could be attributed to this carrier after yeast expression.

Table 1
List of the sucrose transporter sequences available in databases

Name	Species	Length (a.a.)	Accession number	Functional expression/K_m	Ref.
AgSUT1	Celery	512	4091891	Yes/0.14 mM	Noiraud et al., unpublished
AtSUC1	*Arabidopsis*	513	481132	Yes/0.45 mM	[38]
AtSUC2	*Arabidopsis*	512	407092	Yes/0.53 mM	[38]
AtSUTX1	*Arabidopsis*	474	2160188	No	Vystskaia et al., unpublished
AtSUTX2	*Arabidopsis*	513	3287687	No	Rousley et al., unpublished
AtSUTX3	*Arabidopsis*	492	3810593	No	Rousley et al., unpublished
AtSUTX4	*Arabidopsis*	594	3461813	No	Vystskaia et al., unpublished
BvSUT1	Sugar beet	523	633172	No	Westram et al., unpublished
DcSUT1a/b	Carrot	501	2969889, 2969887	Yes/0.5 mM	[64]
DcSUT2	Carrot	515	2969884	Yes/0.7 mM	[64]
LeSUT1	Tomato	511	575299	No	Buerkle and Frommer, unpublished
NtSUT1	Tobacco	507	575351	No	[69]
NtSUT3	Tobacco	520	149981	No	[68]
OsSUT1	Rice	537	2723471	Yes/ND	[71]
PmSUC1	*Plantago*	510	1086253	Yes/0.3 mM	[39]
PmSUC2	*Plantago*	510	415988	Yes/1 mM	[36]
RcSCR1	*Ricinus*	533	542020	Yes/2 mM	[65]
SoSUT1	Spinach	525	549000	Yes/1.5 mM	[26]
StSUT1	Potato	516	542087	Yes/1 mM	[27]
VfSUT1	*Vicia faba*	523	Z93774	Yes/1.4 mM	[67]

Sequences are listed in alphabetical order and, when they are successfully expressed in yeast, the K_m value for sucrose is indicated. ND, not determined.

2.3. Efflux carriers

Although several descriptions of sucrose efflux activities have been reported (e.g., [31]) no such carrier has been identified so far. Some authors have proposed that the influx sucrose carrier could function as an efflux carrier without energisation of the transport, as sucrose would be transported along its concentration gradient [32]. Some data demonstrate the possibility that sucrose transport can occur in the absence of a proton gradient in plasma membrane vesicles from potato [28]. These data could explain the expression of sucrose carriers in the phloem of sink organs [33,34], the high concentration of sucrose in the phloem driving its own efflux outside the conducting tissues. The existence of a carrier involved in sucrose unloading has been postulated from models designed to test the Münch–Horwitz theory [9], indicating that the exit of sucrose has to be rate-limited in order to maintain the movement of the phloem sap. However, this could hold true only if the proton gradient across the plasma membrane is lower than at the loading site.

While studying the uptake of sucrose in plasma membrane vesicles from potato plants where the expression of the sucrose carrier was lowered, it was shown that mesophyll cells are able to take up sucrose [28] confirming data obtained on *Ricinus* cotyledons [35]. However, this could be considered as a retrieval mechanism to pump back sucrose leaked out the mesophyll cells. Therefore the possibility remains that a sucrose carrier is present in the plasma membrane of mesophyll cells, but in the immediate vicinity of the conducting cells, where efflux is occurring. The sucrose concentration gradient across the membrane of such cells (50 mM sucrose inside, less than 1 mM outside [32]) indicates that a facilitator system is capable of allowing sucrose efflux down its gradient, while the very efficient transport system present in the phloem cells keeps the apoplasmic concentration low. In theory, the efflux of sucrose could occur by a proton antiport system, but the direction of sucrose concentration gradient (high sucrose in the cell vs. low sucrose in the apoplasm) is not in favour of an energised step for efflux. However, no such system has been identified in plants so far.

3. Function of the carriers: old and new data

For the rest of this review, only sucrose/H^+ cotransporters will be considered. The most widely used heterologous expression system to characterise those carriers up to now is the yeast *Saccharomyces cerevisiae*. The first sucrose carriers have been isolated by complementation of an invertase deficient yeast mutant [26,27]. Yeast cells have been widely used as they present several advantages for heterologous expression: transformation with foreign cDNAs is routine and very efficient, and growth and processing of cells for sugar uptake measurements are quite simple. For these experiments, minimal material is required and is basically accessible to any laboratory licensed for using radioactive compounds. Moreover, basically any yeast strain is suitable as expression of the endogenous secreted invertase is repressed when glucose is used as the sole carbon source [36]. Sucrose carriers seem to function as monomers in the membrane and are, at least in part, correctly targeted to the plasma membrane in yeast (see [14] for a discussion of this point).

In order to demonstrate that SoSUT1 (the first identified sucrose carrier [26]) was the sucrose transporter extensively studied in plants, several of the properties described in plants were investigated during expression in yeast. Those properties were of three kinds: symport with proton, inhibition by specific reagents and specificity towards sucrose.

3.1. SUTs are proton/sucrose transporters

The first property was demonstrated indirectly by the use of several compounds that collapse the plasma membrane proton gradient established in yeast as in plant cells by a H^+/ATPase of the P-type (for a review see [37]). Therefore protonophores (CCCP, DNP) and inhibitors of ATP generation (antimycin and arsenate) were shown to strongly inhibit the uptake of sucrose into yeast cells expressing SUT [26,38]. Moreover, all SUT carriers successfully expressed in yeast (Table 1) display an optimum activity for external acidic pH (4.5–5.5). However, PmSUC1 and AtSUC1 are less sensitive than other carriers to a rise in pH, having a rather constant activity between pH 4.5–6.5 [38,39]. Sucrose uptake

was also stimulated after addition of glucose to the yeast cells because of glucose stimulation of the H^+/ATPase activity. All these features were in perfect accordance with the properties of sucrose carriers whether studied in leaf discs [40], protoplasts [41] or plasma membrane vesicles (reviewed in [15]). However, more precise data on the kinetics and stoichiometry of the cotransport could only be obtained by oocyte expression [42] as yeast is not suitable for electrophysiological measurements.

As for sucrose transport activities recorded in plants, SUTs were shown to be strongly inhibited by reagents of thiol groups such as PCMBS (para-chloro-mercury-benzene sulfonic acid) and NEM [26,38], and histidine reagents such as DEPC (di-ethyl-pyrocarbonate). The histidyl residue involved in DEPC binding has been determined [43] (see below).

3.2. *Specificity of SUTs. Affinity for sucrose*

When expressed in yeast, all the sucrose carriers display an affinity for sucrose in the range 0.3–1.5 mM. A slightly higher affinity for sucrose was noted for AgSUT1, the sucrose transporter for celery (N. Noiraud, S. Delrot, R. Lemoine, Plant Physiology, in press). Nevertheless, all K_m determinations were not made with the same yeast strains and the uptake conditions might have been slightly different: therefore one can consider that these K_m values are remarkably similar. Interestingly, the K_m for sucrose of StSUT1 expressed in yeast is very close to the value determined in plasma membrane vesicles from potato leaves [28] and also similar to the value measured after expression in *Xenopus* oocytes [44]. Similar K_m values were also measured for AtSUC1 expressed in yeast [38] and oocytes [45]. It has to be noted that the affinity of the sucrose carriers for sucrose is much lower than the affinity of the monosaccharide transporter for glucose [11] by more than an order of magnitude.

All these carriers would therefore correspond to the high affinity system described in plant fragments (*Vicia faba* leaf discs [40], sugar beet leaf discs [46]). The existence of a lower affinity uptake system for sucrose has been postulated from the saturation curves obtained by these authors. However, the complexity of the living systems used (several types of cells) may well be responsible for these results, as only one high affinity system (superimposed by a diffusional component) could be shown when homogenous plant tissues (such as phloem strand from celery petioles [47] or plasma membrane vesicles [28]) were used. The lower affinity system may well represent the activity of a different transporter located on a different cell type.

All the results obtained by expressing sucrose carriers cDNAs in yeast are in perfect agreement with the already described characteristics of sucrose carriers in plants. They demonstrate that the heterologous expression in yeast cells does not change these major characteristics (dependence on the proton gradient, sensitivity to inhibitors). They also indicate that the plant sucrose carriers are able to function properly in a different lipid environment. However, many data have been accumulated on the specificity of the sucrose carrier towards other substrates (natural or synthetic) and those data have been used to construct a model for the interaction of the sucrose molecule with its own carrier [21,48]. According to this model the substrate recognition by the sucrose carrier occurs through hydrophobic interaction with the fructosyl moiety whereas the hydroxyl groups at position C-3, C-4 and C-5 of the glucopyranosyl moiety confer the specificity for sucrose recognition. However, more recent data also suggest that the glycosyl C-2 hydroxyl is also involved in the substrate specificity [49].

How do these data compare with the results obtained in yeast? Although no large-scale analysis of the specificity of SUTs expressed in yeast has been done yet, a certain number of data are available and are listed in Table 2. All these data have been obtained by studying the effects of the sugars listed on radiolabelled sucrose uptake into yeast cells expressing a sucrose carrier. Sugars were used at concentrations in excess of 10- to 50-fold the sucrose concentration and in optimally energised conditions (external medium acidified by the stimulation of H^+/ATPase activity following glucose addition). Therefore these data are rough estimations as they do not give any indication on the type of inhibition. On the other hand, those data should be taken as confirmation that the sucrose carriers identified are the one already described in plants. Several disaccharides have been tested on the basis of steric con-

Table 2
Substrate specificity of the sucrose transporters expressed in yeast

Substance	Effect	Transporter
Sucrose	Inhibition (isotopic dilution)	All transporters tested
Glucose	Stimulation through ATPase activation	All transporters tested
Maltose	Inhibition (67%), 100-fold excess	AtSUC1 and AtSUC2
	Inhibition (70%), 10-fold excess	PmSUC2
	Inhibition (10%), 3-fold excess	RcScr1
	Inhibition (63%), 50-fold excess	SoSUT1
	Inhibition (10%), 10-fold excess	StSUT1
Isomaltose	Inhibition (12%), 10-fold excess	PmSUC2
Lactose	None, 100-fold excess	AtSUC1 and AtSUC2
	Inhibition (14%), 10-fold excess	PmSUC2
	None, 10-fold excess	SoSUT1
Raffinose	None, 100-fold excess	AtSUC1 and AtSUC2
	Inhibition (12%), 10-fold excess	PmSUC2
	None, 3-fold excess	RcScr1
	None, 10-fold excess	SoSUT1
Trehalose	Inhibition (16%), 10-fold excess	PmSUC2
	None, 50-fold excess	SoSUT1
	None, 10-fold excess	StSUT1
Melibiose	None, 10-fold excess	PmSUC2
Melezitose	None, 10-fold excess	PmSUC2
Palatinose	None, 50-fold excess	SoSUT1
	None, 10-fold excess	StSUT1
Phloridzin	Inhibition (84%), 10-fold excess	SoSUT1
	Inhibition (87%), 10-fold excess	StSUT1
α-Phenylglucoside	Inhibition (80%), 10-fold excess	AtSUC1 and AtSUC2
	Inhibition (93%), 10-fold excess	SoSUT1
	Inhibition (92%), 10-fold excess	StSUT1
β-Phenylglucoside	Inhibition (80%), 10-fold excess	AtSUC1 and AtSUC2

The results are obtained from the papers listed in Table 1.

straints. Out of these only maltose (a dimer of glucose linked in α1–4) is a powerful inhibitor of sucrose uptake (Table 2). Its anomer isomaltose has no effect. None of the trisaccharides tested (raffinose and melezitose) were inhibitory to sucrose uptake. These data are in perfect agreement with the sugar specificity of sucrose carriers in plants. Interestingly, phloridzin, a specific inhibitor of animal Na^+/glucose cotransport shown to inhibit sucrose transport in plants [50], is also a very good inhibitor of SoSUT1 and StSUT1 activities [26,27]. Finally, α-phenylglucoside, one of the substrates used to demonstrate the hydrophobic interaction between the fructosyl moiety of sucrose with its carrier, also appears to be a very powerful inhibitor of sucrose uptake. However, the inhibition by β-phenylglucoside is in contrast to previously published data [51]. This point might deserve further investigation. All these different data confirm that the properties described for sucrose transport activities in plants are the same as the one of SUT carriers expressed in yeast. As the family of sucrose carriers increases (at least in *Arabidopsis*) it will be very interesting to see whether all carriers are equivalent as far as kinetic properties and affinity for sucrose are concerned.

3.3. Other expression systems: oocytes and proteoliposomes

Even if yeast cells represent the most common system for expressing sucrose carriers, other expression systems have been used. As indicated in the review by Miller [42], several sucrose carriers have also been successfully expressed in *Xenopus* oocytes. Oocytes represent a very powerful system to study the kinetics properties of an electrogenic cotransporter. It

has allowed a precise determination of a $1H^+/1$ sucrose stoichiometry in the case of StSUT1 [44] and AtSUC1 [45]. To date, expression of plant sucrose carriers in the baculovirus/insect cells system or COS cells has not been reported.

One interesting experiment has been the purification and reconstitution of the sucrose carrier in proteoliposomes. Stolz et al. [52] have modified the PmSUC2 protein by adding a biotin acceptor domain at the C-terminus. The modified protein was successfully expressed in yeast cells and biotinylated. This allowed the purification of the carrier after solubilisation. Moreover, the activity of the biotinylated PmSUC2 in yeast was comparable to the unmodified protein. The purified protein was then reconstituted in liposomes together with beef heart cytochrome *c* oxidase [53]. In the presence of ascorbate, a proton gradient (outside acidic) is created. After reconstitution in such liposomes, sucrose uptake could be recorded only after addition of the electron donor system for cytochrome *c* oxidase (ascorbate/TMPD/cytochrome *c*), indicating that this uptake is coupled to the proton gradient. However, the time course for the uptake of sucrose is very slow as no saturation occurs after 2 h. This is much slower than when plasma membrane vesicles from yeast cells expressing PmSUC2 were fused to liposomes without prior purification of the carrier [36]. Due to the small size of liposomes, an efficient uptake system (such as the bacterial ones already studied [53]) reaches internal saturation in minutes. This rather long time for equilibration in plant proteoliposomes either indicates that a very small number of carrier molecules were incorporated per liposome, that the carrier was inactivated to a certain extent during the purification and/or that the lipid composition of the liposome membrane was not optimal. However, the fact that plant sucrose carriers can be expressed successfully in yeast cells somehow moderates this latter point. Nevertheless, reconstitution of sucrose carriers into liposomes can be used in the future for a precise investigation of the lipid environment effects on the carrier activities.

3.4. Sucrose transporter in other species

As already indicated in the introduction, sucrose is of major importance for plants as a specific form of long-distance transport that cannot be replaced by another sugar molecule. This is very unique to plants as in other organisms (yeast and bacteria able to grow on sucrose) sucrose is dispensable. In bacteria, for example, a proton/sucrose permease has been identified [54] and the residues involved in substrate recognition and binding have been demonstrated to be rather well conserved with the *E. coli* lactose permease [55]. Therefore, very little homology with plant sucrose carriers is expected (see below).

In yeast where sucrose is externally cleaved by a secreted invertase, the resulting hexoses are taken up into the cells. However, there has been a debate about the possibility for yeast cells to directly take up sucrose without prior cleavage. This was demonstrated in several papers [56,57]. However, no sucrose carrier cloning has been reported yet although a general α-glucoside H^+-cotransporter (AGT1) able to transport trehalose and several other disaccharides have been reported [58,59]. While AGT1 preferentially transport trehalose, it has been recently shown that sucrose is also transported efficiently [60]. However, no clear sequence homologies could be found with plant sucrose carriers.

On chromosome I of *Schizosaccharomyces pombe*, a translated sequence related to the sucrose transporter family (accession no. Z99165) has been identified. This is in fact the most closely related sequence to plant sucrose carriers (*E* value 7e-30). However, to our knowledge, no function in sucrose transport has been attributed to this gene.

3.5. Localisation of sucrose carriers

As already discussed, all sucrose carriers identified so far are proton-coupled sucrose uptake transporters (entry of sucrose into cells by cotransport with protons) and would therefore be involved in step 3 of Fig. 1. Data obtained from either immunolocalisation, in situ hybridisation and promoter–reporter gene expression gave some indications about the cells where those carriers are expressed. The first in situ hybridisation experiments clearly located the expression of StSUT1 in the phloem of potato leaves and stems [27]. Immunolocalisation studies then demonstrated that in solanaceous species (potato, tomato and tobacco) SUT1 is expressed in the plasma membrane of the sieve tube [61]. Interestingly, the authors

also demonstrated that the corresponding mRNA was also detected in the sieve elements, although these cells are devoid of ribosomes. For a discussion of this point the reader should refer to [14,61]. In *Arabidopsis* and *Plantago*, SUC2 (the orthologue of SUT1) has been located to the plasma membrane of companion cells [62]. At the present time, it is not known whether these different results correspond to differences among species. However, a recent report from the group of Sauer [63] indicated that in *Plantago*, the second sucrose transporter identified SUC1 is expressed in the sieve tubes, mainly in the petioles where it would be involved in the retrieval of sucrose (equivalent to step 3 in Fig. 1, but along the translocation pathway). It is therefore possible that this situation is general and occurs in all plant species.

Most of the data also indicate that the sucrose carriers are expressed in the phloem, all along the translocation pathway [27,34] and may be involved in the unloading of sucrose in sinks. At the present time, no carrier involved specifically in the entry of sucrose in the sink (step 5) has been identified, although such a function has been reported for DcSUT2 which is highly expressed in carrot tap roots but not restricted to phloem cells [64]. However, lower but detectable levels of expression where also found in the leaf lamina. In *Ricinus*, a sucrose transporter is predominantly expressed in seedlings but also detected in source leaves [65,66]. In *Vicia faba* seeds, expression of SUT1 was detected but again not exclusively in this organ [67]. There is only one report [68] indicating that a tobacco sucrose transporter-like cDNA is specifically expressed in the pollen grains during maturation and also in the pollen tubes during germination. As for monosaccharide transporters [11], it is expected that some of the new sucrose carriers that are identified will be shown to be specifically expressed in defined organs or cells.

However, the localisation experiments must be interpreted with caution. Due to the very high level of sequence identity, care has to be taken that no cross-hybridisation occurs between the different sucrose carriers of the same plants. This point has been solved by raising antibodies against the less conserved regions of the protein (C-terminus or central loop). Even in that case, the sensitivity level of these methods may still be a problem, because only a highly expressed carrier will give a signal of sufficient intensity to be considered as being above background with confidence. In the case of potato, the study of radiolabelled sucrose uptake in plasma membrane vesicles from leaves of transgenic plants where StSUT1 expression was lowered [28] indicated that StSUT1 (or a very closely related sucrose transporter) is expressed in mesophyll cells, but at a level too low to be detected by other methods. This confirmed data obtained on *Ricinus* cotyledons demonstrating uptake of sucrose in mesophyll cells [35] and RcSUT1 expression in epidermal cells [66]. A description of the effect of antisense repression of NtSUT1 in tobacco has also been reported [69].

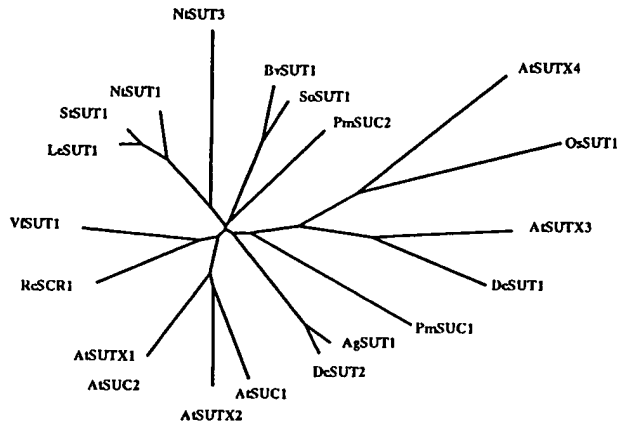

Fig. 2. Phylogenic tree showing the relatedness of the sucrose carriers listed in Table 1, based on the degree of similarity of their sequence. The phylogenic distance is roughly proportional to branch length. The image was generated with the Unrooted software package (pbil.univ-lyon1.fr).

4. Structure/function: the beginning

The number of sucrose carriers identified at the cDNA level is increasing at a fast pace. In the last reviews published on the subject [14,70] 8–12 different sucrose transporters were listed. As seen in Table 1, 20 sucrose carriers are available in the data bases. Two sucrose carriers have been characterised so far in *Arabidopsis* (as in *Plantago*), but it is clear that sucrose carriers make a large gene family and six sequences (Table 1) are present today in the *Arabidopsis* data base. It has to be noted that AtSUC2 and AtSUTX1 differ only by one amino acid. Four of the identified *Arabidopsis* clones cluster together whereas

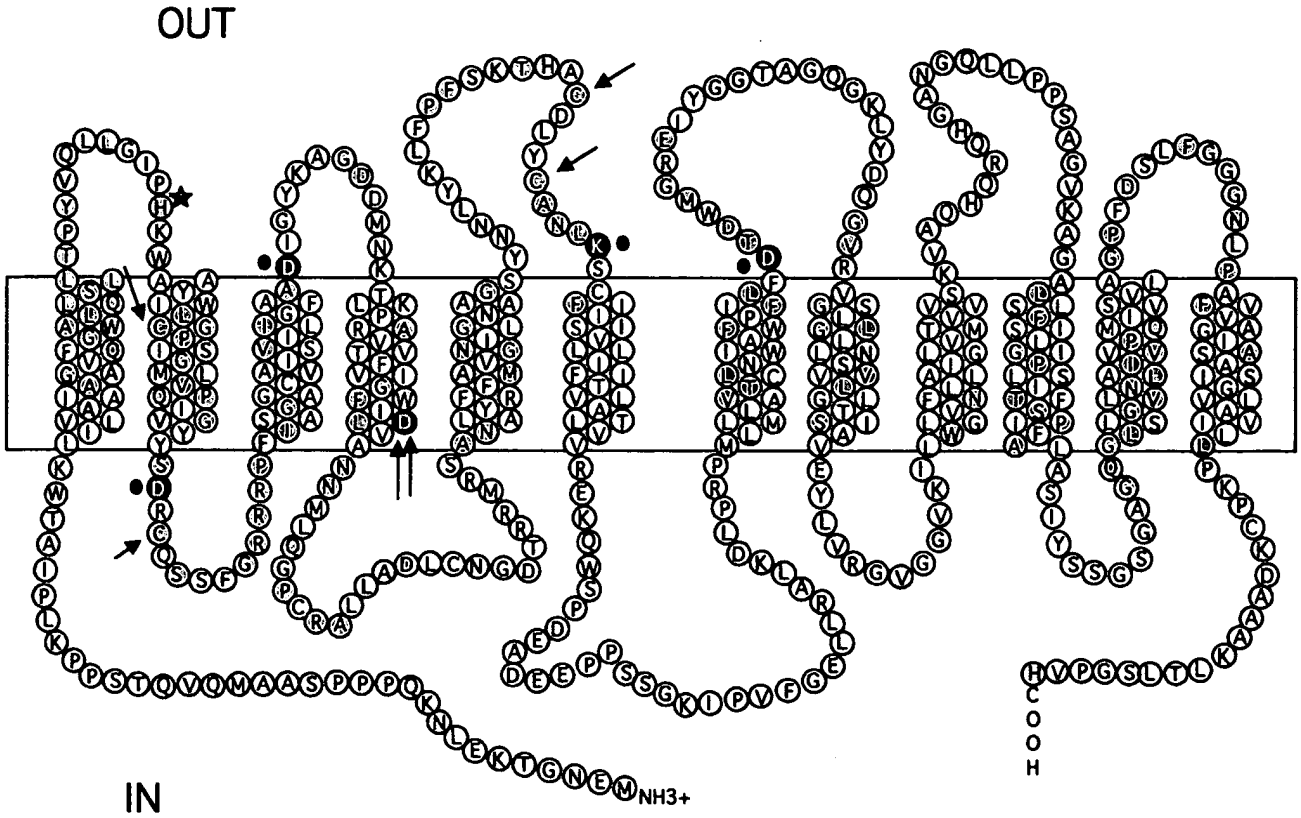

Fig. 3. A tentative prediction for AgSUT1. The transmembrane segments were predicted with HMMTOP [94] after optimisation with all other sucrose transporter sequences available. Sequence alignments were made with the Multialin program at pbil.univ-lyon1.fr [95]. The residues written in green circles are conserved in all 12 sequences shown to function as sucrose transporters (see Table 1) and yellow circles indicate residues conserved in 11 out of 12 of these sequences. Conserved cysteine residues are indicated by arrows whereas points show charged residues located at the border of transmembrane segments (see text). The conserved Asp residue located in a transmembrane segment is indicated by a double arrow. The histidine residue that has been the subject of mutagenesis studies [43] is indicated by a star.

the other two are distantly related (Fig. 2).The next challenge will be to characterise these different clones at the function level. However, for the rest of this review, we will consider the transporters that have been functionally demonstrated to be sucrose carriers (already 12 different clones, see Table 2) by expression in yeast. Since the cloning of the sucrose carrier from rice (OsSUT1 [71]), both monocots and dicots are represented in this list. All these carriers are sequence related (see Fig. 2). The two most distant sequences (OsSUT1 and NtSUT3) still show 37% identity at the protein level. However, DcSUT1 which is located on the closest branch of the unrooted phylogenic tree, already shows 45% identity with NtSUT3.

Although no structural data are available at the present time for any sugar transport protein from any species, one can expect to get valuable information from the sequence comparison of all those 12 well-characterised sucrose carriers. In Fig. 3, a 12-transmembrane α-helices model (two units of six transmembrane segments connected by a central loop) is proposed for AgSUT1. Green circles represent residues that are conserved in all 12 sequences and yellow circles correspond to residues conserved in 11 out of 12 sequences.

As already noted, there is a high conservation of the sequence: 134 conserved amino acids for an average sequence length of 517 which represents 26% of overall conserved amino acids. If the positions where

the residue is conserved in 11 out of 12 sequences compared (yellow in Fig. 3), then 188 residues are conserved (36%). The residues in the first half of the protein are slightly more conserved (30% identity in all 12 sequences, 39% identity in 11 out of 12 sequences) but it is not known if this is relevant to function. The residues predicted to be present in transmembrane regions appear to be more conserved as 79 (out of 250 residues present in putative transmembrane segments) are identical in all 12 sequences compared (32%) and this increases to 43% if one considers residues that are identical in 11 out of 12 sequences. If one would take into account amino acids with similar function, the score would be of course higher as in many positions of putative transmembrane segments, the hydrophobic nature of the residue is conserved among all sequences. Therefore, amino acid conservation occurs in regions which could be related to transmembrane α-helices, whereas a high variability was found in the N- and C-termini and the central loop. The highest conservation is found is transmembrane segments 1, 2 and 11.

4.1. *SUTs are members of the sugar transport superfamily*

Homologies between sugar carriers from eukaryotes and prokaryotes have been described more than 10 years ago [72,73] and this led to the concept of the Major Facilitator superfamily (MFS) [74]. This is a very old family which appeared in prokaryotes more than 3.5 billion years ago [75]. The common feature for members of this superfamily is that they are all supposed to have 12 transmembrane spanning segments in the form of α-helices (based on hydropathy calculations). It has been proposed that those transporters arose from the duplication and fusion of a primordial gene coding a protein with six transmembrane segments.This explains the presence of conserved motives in both halves of the transporters (see below). Moreover, the fact that the two halves of the *E. coli* lac permease (a member extensively studied of the MSF) can be expressed from different promoters and assemble into the membrane to form a functional transporter is in favour of the duplication theory. Members of this superfamily can be uniporters, symporters or antiporters and are able to transport a variety of metabolites. This superfamily has been divided into five clusters [74] and the plant sucrose transporters (as well as plant monosaccharide transporters) are included into the second cluster composed mainly of sugar uniporter from animals, glucose cotransporter from yeast and plants and sugar/H^+ cotransporter from bacteria. It has to be noted that the *E. coli* lactose permease, which is the cotransporter for which most information is available (see below), is not a member of this cluster. Extensive sequences analysis and comparison between the different proteins of the same cluster has led to a proposed conserved motif that could be used as a signature for the members of this cluster. The initial pattern proposed for the sugar transporter was R-X-G-R-[KR] (the first one is located in the loop between the second and third transmembrane domain whereas the second between transmembrane domains 8 and 9). However, this model has been refined (see ProSite 00216) and now includes extension to this initial motif. The pattern located between transmembrane segment 8 and 9 has been extended to the following: [LIVMSTAG]-[LIVMFSAG]-x(2)-[LIVMSA]-[DE]-x-[LIVMFYWA]-G-R-[RK]-x(4–6)-[GSTA] which gives in plant sucrose transporters: [MSTA]-[S]-x(2)-[LIVM]-[EYQD]x-[LIMF]-[GCAV]-[RK]-x (3)-[GA].

This indicates that the second pattern is not heavily conserved in sucrose carriers. Moreover, a third pattern has been described in the loop between transmembrane segments 4 and 5 but this pattern could not be identified among SUT sequences. It has to be noted that the sequences used to design those consensus patterns included only one plant sucrose carrier (SoSUT1). Due to the increasing number of plant sucrose carriers available, the consensus patterns will certainly be changed to take these sequences into consideration. These conserved sequences have not been related to any particular function, except for the small pattern between the second and third transmembrane segments which is predicted to form a β-turn linking the two α-helices [73].

At the present time, no three-dimensional structure is available for any transporter in the MFS superfamily and therefore all the models proposed are hypothetical. Nevertheless, as pointed out by Tanner and Caspari [76], this did not preclude the collection of an impressive number of data leading to stimulating models. The model in Fig. 3 represents the Ag-

SUT1 transporter as a succession of α-helices spanning the membrane, with the N- and C-termini on the cytoplasmic side. What are the basis for such a representation, commonly used for sugar carriers? Hydropathy analysis indicates the succession of hydrophobic and hydrophilic regions. One example is given in [26]. In the case of sucrose carriers, 12 hydrophobic segments can be identified with reasonable confidence, except in the case of the first two transmembrane segments which appear as a large hydrophobic region. The hydropathy profile for SoSUT1 can be taken as a model for all other sucrose carriers known to date. One constraint is that the segments have to be long enough to cross the membrane, which, in the case of an α-helix, is a minimum of 20 residues. However, it has to be kept in mind that an α-helix is not necessarily perpendicular to the membrane as demonstrated in the case of G-protein-coupled receptors [77]. The length of the transmembrane segments determines the number of residues in the connecting loops. This can also give some indications on the arrangement of the different α-helices in the membrane. As pointed out in [78], a short loop will connect two adjacent transmembrane segments whereas longer loops could indicate that two segments that are adjacent in the sequence could be far apart in the tertiary structure. These authors present a general model for members of the MSF, based mainly on the data obtained on the lac permease of *E. coli*, in which helices 2, 3 and 4, 5 and 6; 8, 9 and 10; 11 and 12 are adjacent. From the model presented here and in other papers for sucrose carriers [14], it is possible to hypothesise that helices 2, 3 and 4 are adjacent and so are helices 11 and 12. However, the loop between helices 1 and 2 is certainly short as indicated in the model proposed for StSUT1 [14] because of the high overall hydrophobicity of this region. It is therefore very speculative at the present time to decide whether the sucrose carrier fit into the model proposed in [78]. A recent model for the animal glucose transporters [79] displays a long connecting loop between helices 1 and 2, almost as long as the central loop, a quite different situation from what is seen in the plant sucrose transporters.

As for the orientation of the protein in the membrane, there is now a general consensus to consider that the N-terminus, the central loop and the C-terminus are all located on the cytoplasmic side. This prediction is based on data obtained from studies on the lac-permease and GLUT1, the glucose transporter from human erythrocytes. At the present time no result contradicts this prediction. Such a prediction will be quite easy to verify by challenging antibodies against the N- or C-terminus, with plant plasma membrane of defined orientation (either right-side out or inside out). This could also be useful to identify which residues are readily located into the membrane (but with some caution in the data interpretation as the epitope recognised by the antibodies (monoclonal) has to be precisely known).

Although there is a general consensus on the α-helix nature of the transmembrane segments, some authors have proposed a different model (consisting of a beta barrel) for the structure of GLUT1 (human glucose transporter) and other known sugar carriers [80]. This controversy will be resolved when the three-dimensional structure of such a protein will be determined.

Apart from being related to members of the sugar transporter cluster, sucrose carriers are not directly sequence related to other carriers. For example, very few common elements exist with the hexose carrier plant gene family [11], with an average of 20% of identical amino acids between sucrose and hexose plant transporters [81]. A conserved stretch of residues common to plant sucrose carriers and melibiose carrier of *E. coli* has been described by Naderi and Saier [82]. Three plant sucrose carrier sequences were used to construct this alignment. The same alignment still hold true when more sucrose carriers sequences are included (data not shown). This region is centred on the first conserved motif of the sugar transport family and comprises residues from the second and third transmembrane segments and the interconnecting loop. Interestingly, this region includes two Asp residues that have been involved in the cation selectivity of the melibiose permease. The melibiose permease is peculiar because this carrier is able to catalyse melibiose (or other α-D-galactosides) accumulation by using either Na^+, H^+ or Li^+ gradients [83]. At the present time there is no indication of sucrose carriers able to use other cation gradient than the proton gradient to drive sucrose uptake. In melibiose permease of *E. coli*, the N-terminal domain has been involved in cation recognition in heli-

ces 2 and 4 [83] whereas in the lac permease, the charged residues involved are located in the second half of the protein (helices 7, 8, 9, 10). In plant sucrose carriers the charged residues that are found in a putative transmembrane segment are in the first half of the protein (see below). This could indicate that melibiose permease could be a better model than the lactose permease for identification of important residues.

Regulation of gene expression by sugars has been extensively studied in the yeast *Saccharomyces cerevisiae*. The number of hexose transporters in yeast is surprisingly high (20) but this feature is more understandable as those carriers display different affinities for glucose, allowing a very rapid adaptation of the yeast to changing external sugar concentration. What is even more interesting in yeast is the identification of two members of the hexose transporter family as glucose sensor (Snf3 and Rgt2, reviewed in [84]). Both carriers display an extended carboxy terminus and this has been related to their function as a sensor. The possibility of finding similar carrier/sensor in plants would open new exciting research area. The conservation of the hexokinase pathway in plant sugar sensing argues in favour of the identification of sucrose sensor/transporter proteins [5,85]. It has to be noted that AtSUTX4 has a longer central loop than other sucrose carriers and a similar feature has been found for a tomato sucrose carrier which colocalises with SUT1 in sieve elements [86]. The authors suggest that this protein could be involved in sugar sensing.

4.2. *Conservation of specific residues and structure/function relations*

As already indicated, many residues are conserved among the SUT sequences identified so far and, for the carriers that have been expressed to date, very similar kinetics (e.g., K_m values) were recorded. The major difference noted is the pH sensitivity of SUC1 and SUC2 in *Arabidopsis* and *Plantago* [38,39] but it is difficult to relate specific residues to the difference in function.

It has been noted for many carriers that the location of cysteine residues is often not conserved, even in member of the same transporter family [87]. This would indicate that, in spite of the sensitivity to thiol reagents such as PCMBS or NEM often reported for transporters, cysteine residues are not essential for the transport function. However, in the plant sucrose transporter family, there are four conserved cysteine residues (indicated by arrows in Fig. 3), three in connecting loops and one in a putative transmembrane segment. This number is quite high. Cysteine mutagenesis and protein chemistry have been extensively and successfully used in the study of different carriers [88]. Therefore, this will certainly be a future direction for the work on sucrose carriers. There are several glycosylation sites that are present in the different sequences but not conserved among them. However, there is no evidence at the present time that sucrose carriers are glycosylated in vivo: when the protein is immunodetected on Western blots [28] it appears as a rather sharp band. Conserved phosphorylation sites can be identified in sucrose carriers sequences, also in non-transmembrane segments. The relevance of phosphorylation to the activity of sucrose carrier is discussed in this issue [42,89].

In the case of a proton coupled cotransport system, negatively charged residues are expected to be present in the transmembrane regions in order to translocate protons. In the case of the lac permease, Glu325 has been involved in the proton translocation and four negatively charged residues have been proposed to be involved in the energy coupling [90]. In the case of StSUT1, only one charged residue (Asp, negative) has been located to a transmembrane region [14]. This Asp residue is conserved among all sequences as shown in Fig. 3 (fourth transmembrane segment, double arrow). Whereas this is the only charged residue present in a transmembrane segment in StSUT1, AgSUT1 has three other (positive) charged residues. Those charged residues are only located in transmembrane segments 4 and 5 whereas they are located in the second half of the protein in *E. coli* lac permease (helices 7–11). The small number of charged residues supposed to be present inside the transmembrane domains of sucrose carriers may indicate a different energy coupling system. It is also possible that as the model proposed will be refined, some of the charged residues that are located at the border of transmembrane segments will be included into them. This could be the case of the Asp residues indicated by dark circles in Fig. 3.

4.3. Mutagenesis on sucrose transporters: the infancy

Much of the information available on the structure of symporters has been obtained from mutagenesis studies. Two different strategies can be used: site-directed mutagenesis on particular residues (charged ones or residues having specialised function such as cysteine) or random mutagenesis. The first one has been extensively used in the case of the lac permease of *E. coli* and most of the information known on its structure derives from such experiments. Random mutagenesis has been used in the case of the *Chlorella* hexose carrier, with great success [91,92]. In that case, the carrier cDNA is amplified by PCR under conditions leading to mutations into the cDNA. A mutagenised library is constructed and inserted into a vector for heterologous expression in yeast. Transformed yeast cells are then plated and screened on the desired medium. The screening can be done for altered affinity for the natural substrate (by changing the substrate concentration into the growth medium) or for changes in the substrate specificity. Once a clone showing growth on the selective medium is identified, the mutation responsible for the phenotype is found by sequencing. Unfortunately, mutagenesis work on the plant sucrose carrier is still in its infancy and only one paper has been published on this topic [43]. The authors have made site-directed mutagenesis on a histidine residue of the sucrose transporter from *Arabidopsis* AtSUC1. This residue (His65 in AtSUC1) is conserved in all sucrose carriers and is located in the first extracellular loop, close to the start of the putative second transmembrane segment (marked with a star in Fig. 3). The rationale for mutating this special residue is that sucrose carrier activity has been shown to be inhibited by DEPC, a chemical modifier of histidine residues. As this residue is the only conserved histidine in all sucrose carriers, it therefore appeared as a good candidate for mutagenesis. Histidine was replaced by hydrophobic (Gly, Leu), polar (Cys, Asn, Gln, Ser, Tyr) or charged residues (negative Asp, or positive Lys, Arg). All the different mutant carriers appeared to be expressed equally in yeast, except for the His65–Cys which was degraded after translation, maybe because of incorrect folding of the protein. When histidine was replaced by a positively charged residue (either Arg or Lys), sucrose transport activity in yeast was significantly increased. Both the V_{max} (up to 14-fold for the Lys substitution) and K_m values were increased in such mutants. However, the specificity of these mutant carriers towards sucrose was not tested. The possibility remains that the observed increase in the uptake rate and decrease in the affinity are the results of a lost of specificity. This point will deserve further investigations. Replacing His with a negatively charged residue (Asp) led to dramatic decrease in the V_{max} (8-fold), which is in accordance with the other results. However, replacing His65 with non-charged residues such as Gly or Ser did not change the kinetic properties compared to the wild type, indicating that the positive charge is not mandatory for the activity. The role of His65 in the translocation process is not clear at the present time. In the *E. coli* permease, there is one His at position 322 which cannot be substituted for any other residue without loss of cotransport function and which is involved in the stabilisation of the helices involved in lactose transport [90]. Nevertheless, it is clear that His65 is important for the overall activity of the sucrose transporter. However, the yeast expression system, although very powerful to screen for mutants, shows its limits when fine kinetics have to be investigated. One has to remember that mutants of the *E. coli* lac permease have been studied extensively on membrane vesicles. The use of the yeast expression system as a first screen combined with oocyte expression will certainly give invaluable information.

The identity of His65 as the target residue for DEPC was confirmed by the fact that the mutant carriers devoid of His65 and still able to transport sucrose were less or not at all sensitive to DEPC. This first attempt to identify a mutant in the transport pathway of sucrose and proton therefore gave very interesting results and calls for more research being done in that area. Screening for mutants with altered specificity is also important as, for example, in *E. coli*, lac permease have been mutated to transport sucrose with a higher affinity than the wild-type carrier [93].

5. Conclusions and future prospects

The existence of several sequences related to al-

ready known sucrose carriers in the *Arabidopsis* database indicate that a whole family of sucrose transporter genes is present in one single plant. The total number is still unknown as it will certainly increase at the same pace as new sequences are released. The next challenge will be to unravel the exact function of the new genes and their role in the plant. It is reasonable to expect that carriers with different specificities and/or kinetic properties will be identified. Carriers with different affinities for sucrose will certainly be found as different cell types encounter different sucrose concentrations. However, as in the case of hexose carriers, the expression pattern of some of these carriers might be very restricted to certain cell types or developmental phase, making their localisation more difficult. However, the knowledge of all the sequences for sucrose transporters will be of considerable interest for the structure/function studies.

Another challenge will be to understand more about the way plants regulate the flow of sucrose both at the whole plant or cellular level. Whether homologous genes to the yeast glucose sensors (Snf3 and Rgt2) are present in plants is a very stimulating problem for the understanding of gene regulation in plants. This will of course relate to former physiological questions such as the priority among different sinks (see Fig. 1) competing for sucrose delivery and use. These problems are related to plant productivity as a high harvest index is obtained when the harvested organs (sinks) received a significant portion of the exported sucrose. It might therefore be conceivable to modify the flow of sucrose to a particular sink by changing the expression of selected carriers and/or sensors. This future direction of work will be reasonable to follow when all carriers are known and precisely characterised.

The other sucrose transporters involved in different transport events (tonoplast carrier, efflux carrier, sink-specific carriers) will also have to be identified. No indication exists, for example, that the tonoplast sucrose carrier is related in sequence to other sucrose carriers, although there are antiporters that are members of the Major Facilitator superfamily, but not in the same cluster. New methods will have to be designed and used for these identifications.

As already noted, the structure/function studies on the sucrose carriers are still in their infancy. Nevertheless, it is obvious that more information is needed on the residues relevant to some of the properties of the sucrose carrier such as selectivity and affinity. This may not only be interesting from a fundamental point of view but also as a possibility to alter the flow of sucrose to sink, or to alter the selectivity of the carrier so that it would accept foreign molecules (xenobiotics or natural) and allow their long distance transport in the plant. This was in fact one of the original aims for the identification of the sucrose carrier back at the beginning of the 1980s. Improving the quality of sinks is also a positive outcome to be expected. However, there is still much information that needs to be obtained. Of course the main problem is the lack of three-dimensional structure elucidated to date. In spite of efforts from several groups to overproduce membrane carriers, no crystallisation of any transport protein could be obtained. However, many data are still to be collected for reaching a knowledge level similar to the lac permease. A thorough review of the different experiments that led to the partial understanding of the structure/function of the lac permease is presented in [90]. Compared to the lac permease, our knowledge on the sucrose carriers is scarce not only on the structure but also on the function. Thanks to the oocyte expression, precise data are becoming available on the cotransport phase with proton. This system will certainly be invaluable for the study of mutagenised carriers altered in the coupling of the two substrates. Much information is surprisingly lacking on the function of the sucrose carrier in the absence of a proton gradient, its kinetics when the sucrose gradient is inverted (efflux?) and its precise selectivity. The lac permease displays the advantage that it can be overexpressed in its own native lipid environment, which allowed a precise study of all its kinetic characteristics. In plants, however, plasma membrane vesicles studies have not given such a level of information (for a review, see [15]) and the heterologous expression in yeast has been mainly used to confirm the nature of such carriers as sucrose transporters. Therefore, the lack of an expression system as convenient and efficient as *E. coli* might impair some of these studies.

It may still be a long time before a paper devoted to sucrose carrier structure will contain more than speculative data, but the prominent role of sucrose

transport and sucrose transporters for the growth of plants will certainly lead to a dramatic increase of our knowledge in the field.

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CROP PHYSIOLOGY & METABOLISM

Heat Stress during Grain Filling in Maize: Effects on Kernel Growth and Metabolism

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ABSTRACT

The average temperature in the U.S. Corn Belt during the grain-filling period of maize (*Zea mays* L.) is above optimum for maximum grain yield. The objectives of this study were to determine the effects of an extended period of high temperature during grain filling on kernel growth, composition, and starch metabolism of seven maize inbreds. Plants were exposed to heat stress (33.5/25°C) or control (25/20°C) day/night temperature treatments in a greenhouse from 15 d after pollination (DAP) until maturity, and the experiment was conducted in triplicate over time. Root zone temperature was maintained at 25/20°C in both treatments. No significant interaction occurred between genotype and temperature treatments for nine grain traits. Heat stress lengthened the duration of grain filling on a heat unit (HU) basis, but an overcompensatory reduction in kernel growth rate per HU resulted in an average mature kernel dry weight loss of 7% ($P = 0.06$). Proportionally similar reductions occurred for starch, protein, and oil contents of the kernel. Heat stress also reduced kernel density. A survey of 11 enzymes of sugar and starch metabolism extracted from developing endosperm revealed that ADPglucose pyrophosphorylase, glucokinase, sucrose synthase, and soluble starch synthase were most sensitive to the high temperature treatment. However, upon adjusting enzyme activities with measured temperature coefficients (i.e., Q_{10}), only ADPglucose pyrophosphorylase exhibited reduced activity. Results indicate that chronic heat stress during grain filling moderately restrains seed storage processes and select enzymes of starch metabolism to similar degrees across multiple maize inbreds.

THE AVERAGE TEMPERATURE during the grain-filling period of maize in the U.S. Corn Belt is above optimum (22.5°C) for maximum dry matter accumulation in kernels, resulting in a reduction in grain yield (Thompson, 1986). An estimation based on crop production and meteorological records, indicates that a 6°C rise in temperature from 22 to 28°C during grain filling results in a yield loss of ≈10% in the U.S. Corn Belt (Thompson, 1966). In a field study of maize, Muchow (1990) did not see a yield loss associated with high temperature during grain filling. Muchow (1990) found that during five growing seasons in Northern Australia, yield was unaffected by temperatures, which ranged from 25.4 to 31.6°C during the period from pollination to 80% maximum grain size. A growth chamber study by Badu-Apraku et al. (1983) shows a more dramatic yield loss associated with high temperatures during the period of grain filling . They observed a 42% loss in grain weight per plant when day/night temperature from 18 d post-silking to maturity was increased from 25/15 to 35/15°C, a 6°C rise in average daily temperature.

The interaction of heat stress with other environmental factors in the field, such as drought stress, makes it difficult to study the effect of high temperature on maize yield in isolation. Furthermore, it may be difficult to separate the effects of heat stress occurring during grain filling from a previously occurring heat stress. The use of controlled environments makes it possible to study more precisely how high temperature treatment affects maize grain filling. However, controlled-environment studies should strive to mirror conditions in the field as closely as possible. As described below, conditions of root temperature and photyosynthetically active radiation (PAR) intensity can differ between the field and controlled environment and have been shown to be important factors that help determine how plants respond to high temperature.

Roots growing under a canopy of maize plants in the field are well buffered from extremes in air temperature during grain filling (J.K. Radke, 1995, personal communication). The buffering capacity of the soil to extremes in air temperature is well illustrated by Radke's average daily maximum/minimum temperature recordings during the month of August in 1992 and 1994 at the canopy level (29.3/13.9°C) and at soil depths of 5 cm (24.7/17.8°C), 15 cm (21.8/19.3°C), and 50 cm (19.6/19.2°C). In contrast, the root temperature of plants grown in pots will reflect the ambient temperature unless efforts are made to control soil temperature independently of temperature experienced by aerial portions of the plant. Furthermore, roots have been shown to be highly sensitive to high temperature (e.g., >25°C), especially compared with plant shoots. Kuroyanagi and Paulsen (1988) studied the effects of four root/shoot temperatures (25/25, 35/25, 25/35, and 35/35°C) during grain filling on dry weight accumulation of developing wheat (*Triticum aestivum* L.) grains. Grain dry weight of plants with roots held at 35°C was dramatically reduced when compared with plants with roots at 25°C. However, in plants with roots at the lower temperature, a 10°C shift in shoot temperature had a smaller effect on grain growth. Roots of tepary bean (*Phaseolus acutifolius* Gray) and com-

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Abbreviations: AGPase, adenosine diphosphate-glucose pyrophosphorylase; DAP, days after pollination; HU, heat units; PAR, photosynthetically active radiation; SSS, soluble starch synthase.

mon bean (*Phaseolus vulgaris* L.) were also found to be more sensitive to high temperature than plant shoots (Udomprasert et al., 1995).

Plants exposed to the low PAR intensities occurring under growth chamber conditions are less tolerant of high temperature stress. Wardlaw (1970) demonstrated that low PAR intensity during grain filling of wheat significantly reduced grain dry weight, with the effect most pronounced at high temperature. Spiertz (1977) exposed wheat plants grown in a growth chamber to 12 combinations of temperature and PAR following anthesis. The study showed that low PAR intensities decreased grain growth by a greater amount under high temperature than under normal temperature. For the aforementioned reasons, root temperatures and PAR intensity comparable to the field should be incorporated into an experimental design to portray realistic effects of high temperature stress on maize plants.

The mechanisms in cereals that are affected by heat stress and limit kernel growth are not well understood. Mature kernel dry weight is determined by the product of the rate and duration of grain growth, both of which are influenced by temperature (Tashiro and Wardlaw, 1989). Generally, in lower temperature ranges (≈10–25°C), cereals respond to increasing temperature with an increase in the rate of grain filling per unit of time. At temperatures greater than a critical maximum (≈25–35°C), the gain in the rate of grain filling begins to diminish, and at supra-optimal temperatures (≈40–45°C) the grain-filling rate drops precipitously. Rising temperatures also produce a progressive decline in grain-filling duration. Hence, at high temperatures, yield losses are the result of a loss in both grain-filling rate and duration.

Grain-filling duration may be determined by a number of factors including sucrose availability to the kernel (Afuakwa et al., 1984) and activity levels of enzymes involved in sugar and starch metabolism in the kernel (Singletary et al., 1994). Similarly, the rate of grain filling may be affected by sucrose concentration in the kernel (Jenner, 1970) and activity levels of enzymes in the pathway of starch biosynthesis (Jenner et al., 1993; Keeling et al., 1993, 1994).

The effects of temperature on starch biosynthesis in the kernel have received much attention because starch accounts for most of the dry weight in cereal grains. In wheat grains, losses in starch accumulation caused by heat stress are believed to be linked to a reduction in the activity of soluble starch synthase (SSS) (Hawker and Jenner, 1993; Jenner et al., 1993; Keeling et al., 1993). The mechanisms limiting starch synthesis in chronically heat-stressed maize kernels are not so well understood. Singletary et al. (1994) studied 13 enzymes of sugar and starch metabolism in maize kernels grown in vitro and exposed to a range of chronic heat stresses. The activities of ADPglucose pyrophosphorylase (AGPase) and SSS were reduced the most, and their activities were prematurely terminated compared with other enzymes. The authors concluded that reductions in starch synthesis under heat stress are closely tied to the duration of activity for these enzymes. Keeling et al. (1994) assayed 11 enzymes of starch synthesis extracted from kernels exposed to a short-term (3 h) high temperature stress in vitro. The activity of SSS was reduced most by high temperature and reached a maximal rate at 25°C. Other enzymes (with the exception of branching enzyme) increased in activity up to a temperature of 45°C. Reductions in the rate of SSS were similar to losses in the rate of starch synthesis caused by heat stress.

The objective of this study was to extend our understanding of the effects of chronic high temperature during grain filling on kernel growth, composition, and starch metabolism in maize plants. Unlike previous controlled-environment studies, we independently regulated root zone temperatures and PAR to simulate field conditions. To do this, plants were greenhouse-grown during summer and early fall for a high PAR level, and root zone temperature was maintained at day/night temperatures of 25/20°C for both temperature treatments. Seven inbred lines, representing different heterotic groups, were selected for the study.

MATERIALS AND METHODS

Plant Materials and Treatment

Plants of the seven maize inbred lines (ICI04, ICI12, ICI63, ICI66, ICI94, ICI95, and ICI98) were grown individually in 10-L pots in greenhouses at the ICI Seeds research site in Slater, IA and were exposed to a 25/20°C day/night temperature regime prior to initiation of the temperature treatments. Plants were fertilized at planting and at 30-d intervals thereafter with 15 g of controlled-release 19-6-12 Osmocote (Grace Sierra, Milipitas, CA), 5 g of 0-46-0 (Lange-Stegman, St. Louis, MO), 1.67 g of 0-0-62 (Lange-Stegman, St. Louis, MO), and 1.6 g of Sprint 330 (10% chelated Fe) (Ciba-Geigy, Greensboro, NC) per pot. To prevent the growth of soil pathogens, 0.225 g of Banrot (5-ethoxy-3-trichloromethyl-1,2,4-thiadiazole and thiophanate-methyl) suspended in 0.5 L of water was applied to each pot at 3 and 6 wk after planting. Sibling pollinations were performed at 1 and 3 d following the extrusion of silks, and only plants with well-filled ears were used in the temperature treatments. Plants were kept well watered and relative humidity was maintained between 50 and 80% to avoid drought stress. The two temperature treatments began at 15 DAP and lasted until maturity. At 15 DAP, six plants of good health were chosen from each inbred line and divided equally between a high temperature (mean daily temperature of air: 33.5/25°C, and root zone: 25/20°C) greenhouse and an adjacent control (mean daily temperature of air: 25/20°C, and root zone: 25/20°C) greenhouse (Fig. 1). Temperature treatments were based on a 16-h photo/thermal period. They are hereafter referred to as 34/25°C and 25/20°C. The high temperature treatment represented a chronic heat stress of greater duration than normally experienced in the U.S. Corn Belt, but which may occur in maize producing areas in the Southern United States. The high temperature treatment was established to produce a noticeable temperature effect while remaining in a range of agronomic importance. The control treatment was representative of a near-optimal grain-filling temperature (Thompson, 1966). Root zone temperature was established at 25/20°C on the basis of estimates of actual root zone temperatures that normally occur under a canopy of maize during grain filling (see above).

Root zone temperature was controlled by growing plants in specially designed "rootboxes" constructed of two layers

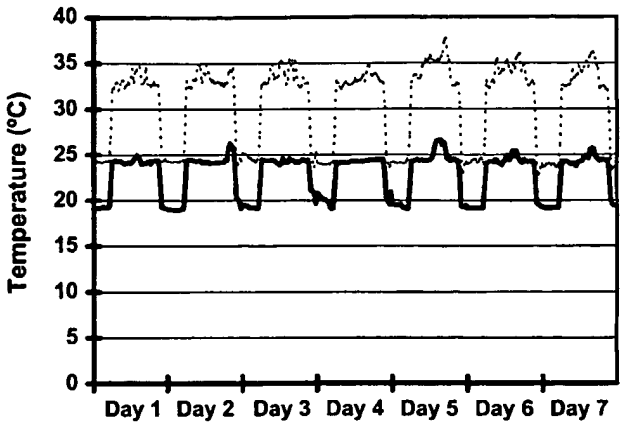

Fig. 1. Air temperature measured during a typical week in the high temperature (dotted line) and control (solid line) greenhouses.

of 5-cm-thick polystyrene insulation boards fashioned into a 3.5 by 0.6 by 0.6 m rectangular box. The lids of each box had eight 10-cm-diameter openings that allowed the aerial portion of the plant to grow outside of the box. Lids were divided in half for access to the pots. On one end of each rootbox was a 15-cm-diameter opening for circulating air into the box. Air from the control house was delivered to rootboxes in both the control house and the adjacent high temperature house through a manifold located in the control greenhouse. The manifold was a 2 by 0.6 by 0.6 m box, constructed of the same material used for rootboxes, with five blowers mounted on top. The manifold was connected to rootboxes with insulated tubing. In both greenhouses, a sensor to monitor air temperature was placed at ear height and a sensor to monitor soil temperature was buried at the 5-cm depth in one pot. The sensors were connected to a LI-COR (LI-COR, Lincoln, NE) model LI-1000 data logger.

Analyses of variance were conducted for each variable of response in a split-plot experimental design. The main plot was a greenhouse divided by a glass wall into a control temperature room and a high temperate room. Temperature treatments (two) were assigned to the main plots, and inbreds (seven) were assigned to subplots. Subplots consisted of three plants per inbred, and analyses of variance were determined using average values of the three plants. Plants within each temperature treatment were randomly placed and rerandomized weekly. The main plots were replicated across three planting dates in 1994: 6 May, 22 June, and 27 July. A total of 123 plants were used in this study.

Kernel Sampling and Analyses

Kernels were collected from maize ears at three dates during grain filling determined by the accumulation of HU following pollination (Table 1). The daily accumulation of HU was calculated with the following formula:

$$\text{Accumulated HU} = (\text{ADT} - 10°\text{C})D \qquad [1]$$

where ADT is the average daily temperature recorded on an hourly basis (constant during the experiment), 10°C is the base temperature for maize growth (Cross and Zuber, 1972), and D is the accumulation of time in days. A fourth and final harvest was collected following black-layer formation.

Kernels were collected according to the procedure of Duncan and Hatfield (1964). For each sampling date, at ≈8 h into the light cycle, a strip of husk was peeled from the proximal end of the ear to expose two rows of kernels, and 10 to 15 kernels were removed from a single row in the central portion

Table 1. Kernel collection schedule. For both treatments, harvest times were based on the accumulation of heat units (HU) following pollination.

Harvest	Accumulated HU (both treatments)	Days after pollination 25/20°C	34/25°C
1	190	15	15
2	250	20	18
3	320	25	21
4	maturity	maturity	maturity

of the ear. Kernels were immediately frozen in liquid N and stored at −80°C. Ears were sprayed with 0.440 g L^{-1} Benlate 50W (methyl 1-[(butylamino)carbonyl)]-1H-benzimidazol-2-ylcarbamate(CA)) fungicide to prevent fungal growth, and the husk was returned to its original place and secured to the ear with rubber bands.

The rate and duration of grain filling was calculated according to Johnson and Tanner (1972). Rate was calculated on the basis of HU (mg $kernel^{-1}$ HU^{-1}) and on the basis of time (mg $kernel^{-1}$ d^{-1}). Duration was expressed as total HU accumulated and as total days.

Density of mature grains was measured with a multi-pycnometer, model MVP-1 (Quantachrome Corp., Syosset, NY). Protein, oil, and starch concentrations of mature kernels were determined with an Instalab 600 NIR product analyzer, model 15299-1810V5 (Dickey-John Corp., Auburn, IL).

Enzyme Extraction and Assay

All chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, MO. The radiochemical adenosine diphospho-D-[U-^{14}C]Glc (1.06×10^{13} Bq mol^{-1}) was obtained from Amersham Corporation, Arlington Heights, IL. All tissue used in enzyme assays was from kernels collected at the second harvest (250 postpollination accumulated HU). After removing the pericarp and embryo from frozen kernels with forceps, the endosperm was lyophilized, finely ground in liquid N with a mortar and pestle, and returned to −80°C.

The enzyme extract was prepared by adding 100 mg of endosperm tissue to 2 mL of extraction buffer [50 m*M* Hepes-NaOH (pH 7.5), 5 m*M* $MgCl_2$, 1 mg mL^{-1} bovine serum albumin, 1 m*M* dithiothreitol], and homogenizing at 25 000 rpm for 20 s (PowerGen 700, Omni International, Warrenton, VA). The homogenate was centrifuged (30 000 × g, 20 min., 4°C) and the supernatant saved for assay of soluble enzymes. The activities of ATP-dependent fructokinase, UTP-dependent fructokinase, glucokinase, and sucrose synthase were measured in dialyzed extracts, with dialysis conducted overnight in extraction buffer at 4°C.

Assays used to measure ATP-linked fructokinase, UTP-linked fructokinase, glucokinase, sucrose synthase, PPi-linked phosphofructokinase, ATP-linked phosphofructokinase, and AGPase were described by Singletary and Below (1990), with the modification of 40 m*M* UDP for the sucrose synthase reaction mix. Phosphoglucomutase, phosphoglucoisomerase, and UDPglucose pyrophosphorylase were assayed by the procedures of Doehlert et al. (1988) using 100 mM Hepes (pH 7.7), 1 m*M* UDP-Glc, 50 μ*M* Glc-1,6-bisphosphate, and 2.5 U mL^{-1} of the coupling enzymes. With the exception of sucrose synthase, these enzyme activities were measured by mixing reactions in a microtiter plate and monitoring the change in absorbance at 340 nm with a BIO-TEK Instruments (Winooski, VT) Model EL 340 automated microplate reader. Substrate-independent background rates were measured and subtracted from the complete assay to obtain the enzyme activity. To minimize variability between triplicate assays run in micro-

titer wells, Tween-20 (2×10^{-5} mL mL^{-1} final concentration) was included in reaction mixtures.

The activity of SSS was determined using 50 μL of undiluted extract as part of a 200-μL reaction mix [100 m*M* Bicine (pH 8.3), 500 m*M* sodium citrate, 5 m*M* EDTA (ethylenediaminetetraacetic acid), 10 m*M* glutathione, 10 mg mL^{-1} rabbit liver glycogen, and 1 m*M* ADP-Glc (^{14}C), 220 disintegrations min^{-1} nmol^{-1}]. Radiolabeled ADP-Glc was added to start the reaction. The reaction was stopped at 10 min by boiling for 2 min. Unreacted ADP-Glc [^{14}C] was separated from radiolabeled glucan by passing the reaction mix through OH^- ion exchange resin columns as described by Jenner et al. (1995). Radioactivity was measured using a Beckman (Fullerton, CA) model LS 5000TD scintillation counter. Background counts were determined by running the assay with boiled extract.

Sucrose synthase and SSS assays were performed in duplicate. Enzyme activities of both temperature treatments were measured at 25°C and reported. For the high temperature treatment, values were also reported after adjustment with the temperature coefficient (Q_{10}) of respective enzymes to estimate the in vivo catalytic activity in heat-stressed kernels. The Q_{10} adjustment was made according to the following formula (adapted from Hochachka and Somero, 1973):

$$VE_{31} = (V_{25})Q_{10}^{(31-25)/10} \quad [2]$$

where VE_{31} is an estimate of the enzyme activity at 31°C (the average daily temperature in the high temperature treatment) and V_{25} is the measured enzyme activity. The individual enzyme Q_{10} values used in this calculation were obtained from Wilhelm (1995) using the average Q_{10} value measured in 5°C increments across the range of 20 to 35°C (Table 2). Enzyme activity is reported in units with one unit of enzyme activity defined as the formation of 1 μmol of product per minute at 25°C.

RESULTS AND DISCUSSION

Kernel Growth

Analysis of variance of the split-plot design reveals that there were significant treatment responses to temperature ($P \leq 0.09$) and inbred ($P \leq 0.04$) for all grain quality and growth parameters measured (Table 3). This verifies that the contrasting temperatures and germplasm chosen for this study produced a notable impact on maize seed growth and development. In contrast to temperature and inbred effects, tests for significance of the inbred × temperature interaction revealed *P* values >0.10, except for grain-fill rate (Table 3). Hellewell et al. (1996) also noted that kernel growth of oat (*Avena sativa* L.) cultivars responded the same to contrasting temperature regimes. Nevertheless, the absence of a genotype × temperature interaction in our study and in that by Hellewell et al. (1996) is more the exception than the rule. Cultivars of wheat (Wardlaw et al., 1989a, 1989b; Wardlaw and Moncur, 1995), barley (*Hordeum vulgare* L.; Savin et al., 1996), and rice (*Oryza sativa* L.; Yoshida and Hara, 1977) commonly display differences in seed growth under conditions of supra-optimal grain fill temperatures as opposed to normal temperatures. The same has also been reported for maize. Lu et al. (1996) exposed developing maize kernels to high temperature stress independently of the rest of the plant and found a striking difference in growth response of different inbreds. We believe that in making genetic comparisons related to kernel heat-stress tolerance it is important to recognize that temperature stress applied in different fashions can produce disparate degrees of genotype sensitivity. For instance, maize kernels of eight inbreds displayed similar levels of growth susceptibility to high temperature when grown in vitro (Singletary and Banisadr, 1992), but when plants of the same inbreds were raised in growth chambers and heat stressed during grain filling (25 vs. 35°C; root temperature not controlled) genotypic differences in kernel heat-stress tolerance were much more pronounced. Hence, in view of mixed results that are reported for genotypic heat-stress tolerance, it is important to emphasize that the results of our study do not rule out the possibility that developing kernels of some maize genotypes may tolerate high temperatures better than others. Nevertheless, because the inbreds we chose represented several heterotic groups and maturities, and because of the difference in the way root temperature was controlled in our work vs. other indoor studies with maize (see discussion below), we are led to conclude that the effect of heat stress on developing kernels of maize probably does not differ widely among elite inbreds.

Kernel dry weight was reduced an average of 7% under high grain-fill temperature (Table 4). Such losses would be of considerable economic importance to Midwestern U.S. grain producers. This abatement in seed growth occurred when average daily temperature increased by 7.5°C (23.5 vs. 31°C), and it is indicative of how plants may respond to a similar type and level of stress in the field. In fact, results of our study are comparable with the level of yield loss caused by a 6°C increase in grain-fill temperature during August, as estimated by Thompson (1966) using maize production records for the U.S. Corn Belt. We believe this is because we simulated a heat stress as it would naturally occur in the field by applying heat stress to the entire aerial portion of the plant under natural sunlight conditions, while buffering roots from extreme temperature changes. Maize plants can tolerate moderately high ambient temperatures (35°C) during grain fill and still produce kernels of normal or near-normal size if the rooting profile is not adversely heat stressed (Table 4; Muchow, 1990). Kuroyanagi and Paulsen (1988) demonstrated the adverse effect of high root temperature, as opposed to shoot temperature, on wheat grain growth several years ago, but their work has received little attention and many researchers continue to ignore the impact of tem-

Table 2. Average temperature coefficients (Q_{10}) of maize endosperm enzymes in the range of 20 to 35°C (Wilhelm, 1995).

Enzyme	Q_{10} (20–35°C range)
Sucrose synthase	1.90
UDPglucose pyrophosphorylase	1.52
Glucokinase	1.53
ATP-linked fructokinase	2.62
UTP-linked fructokinase	1.41
Phosphoglucomutase	1.62
Phosphoglucoisomerase	2.35
ATP-linked phosphofructokinase	1.33
PPi-linked phosphofructokinase	0.97
ADPglucose pyrophosphorylase	1.32
Soluble starch synthase	1.33

Table 3. Summary of analysis of variance mean squares (MS) and corresponding *P* values (in parentheses) of nine grain traits for the inbred, temperature, and inbred × temperature (I × T) sources of variation.

Grain trait	Source of variation					
	Inbred		Temperature		I × T	
	MS	*P*	MS	*P*	MS	*P*
Dry weight, mg kernel^{-1}	1563	(0.01)	1751	(0.06)	95	(0.91)
Starch content, mg kernel^{-1}	536	(0.01)	642	(0.07)	37	(0.94)
Protein content, mg kernel^{-1}	65	(0.01)	62	(0.06)	4	(0.64)
Oil content, mg kernel^{-1}	10.7	(0.01)	3.2	(0.06)	0.2	(0.91)
Density (× 10^{-4})†	43	(0.01)	52	(0.09)	2	(0.57)
Grain fill rate, mg kernel^{-1} day^{-1}	4.8	(0.01)	21.6	(0.01)	0.6	(0.12)
Grain fill rate (× 10^{-3}), mg kernel^{-1} HU^{-1}‡	17	(0.01)	178	(0.01)	2	(0.09)
Grain fill duration, d	23	(0.04)	318	(0.01)	4	(0.81)
Grain fill duration, HU	7336	(0.01)	52047	(0.01)	831	(0.87)

† Actual values equal reported value multiplied by the number in parentheses.
‡ HU = heat unit.

perature treatments on roots. The low PAR under artificial lighting may also reduce the ability of plants to tolerate higher temperatures (Spiertz, 1977). Thus, the large effect (nearly 50% reduction) of high temperature on maize kernel dry weight as found in previous controlled-environment studies performed under artificial lighting and without root-temperature control (Badu-Apraku et al., 1983; Singletary and Banisadr, 1992) may be misleading. Based on these studies and our data, we hypothesize that proper control of root temperature and lighting is needed when studying the effects of heat stress on aerial portions of the plant. Likewise, we theorize that heat stress is more harmful to kernels treated in vitro than for those stressed as part of the aerial portion of the plant. Singletary et al. (1994) and Duke and Doehlert (1996) found that the deposition of starch, the primary constituent of the seed, was reduced 20 to 45% for maize kernels grown in vitro at 30 vs. 25°C. Our data show that growth of kernels experiencing a daily average temperature of 31 vs. 23.5°C was only reduced by 7% (Table 4). For wheat, Hawker and Jenner (1993) found that continuous heating of intact wheat heads at 35°C (vs. 19°C) for 7 d reduced kernel dry weight 11 to 20%, but the rate of starch synthesis in isolated kernels heat stressed in vitro at 35 vs. 20°C was reduced 43% (Jenner et al., 1993). The interaction between plant and seed appears to be important in determining how the seed responds to changes in temperature, and we believe that keeping the seed intact with the whole plant in temperature studies is vital for determining the true effects of temperature stress on the seed.

In addition to evaluating mature kernel mass, we also examined the effect of high temperature on the dynamics of seed growth. Immature grain in both temperature treatments was collected after the accumulation of a specific number of HU in order to isolate kernels at equivalent stages of physiological development. Kernel growth was also measured as a function of time to compare with previously published work. In the latter regard, the rate of grain filling per day was increased 19% and the day-based duration of grain filling was reduced 22% at high temperatures (Table 5). This agrees with previously reported work in maize (Tollenaar and Bruulsema, 1988; Muchow 1990), rice, and wheat (Sofield et al., 1977; Tashiro and Wardlaw, 1989). Interpreting the rate and duration data on a day basis suggests that high temperature reduces kernel size through a reduction in the duration of the fill period and the lack of a compensatory increase in grain-filling rate. However, the importance of the effects of high temperature on the efficiency of fill rate may equally explain why high temperature reduces final seed dry weight. This is illustrated when rate and duration of grain filling are calculated on a HU basis. The HU basis was used to put the data on a normalized basis, inasmuch as temperature, not days, is the primary factor that drives the progression of crop and seed growth. Thus, on an accumulated HU basis, high temperature reduced the rate of grain filling 24% and increased the duration of dry matter accumulation 21% (Table 5). On this basis, the larger reduction in rate of grain filling, as compared with duration, was responsible for the heat-related reduction in seed mass. These heat unit–based responses are in contrast to what we have observed with maize kernels grown in vitro. In culture, heat stress has a small effect on the rate of kernel growth per HU and instead largely causes a premature termination in the duration of storage product deposition (Singletary et al., 1994). However, the data from our study and published data (Wardlaw and

Table 4. Mature kernel dry weight, starch, protein, and oil content across all genotypes as affected by two temperature regimes during grain filling.

Grain trait	25/20°C	34/25°C	Difference	
	mg kernel^{-1}		%	*P*
Dry weight	180	168	−7	0.06
Starch content	120	112	−7	0.07
Protein content	24.4	21.9	−10	0.06
Oil content	7.4	6.9	−7	0.06

Table 5. The rate and duration of dry matter accumulation in the kernel across all genotypes as affected by the two temperature regimes during grain filling.

Grain filling characteristics	25/20°C	34/25°C	Difference
			%
Grain fill rate, mg kernel^{-1} HU^{-1}	0.55	0.42	−24**
Grain fill duration, HU	333	403	21**
Grain fill rate, mg kernel^{-1} d^{-1}	7.4	8.8	19**
Grain fill duration, d	24.4	19.4	−22**

** Significant at $P \leq 0.01$.

Moncur, 1995) converted to a HU basis show that when maize and wheat plants experience supra-optimal temperatures during grain fill, the rate of seed growth is decreased and the duration is increased, but the increase in duration is not large enough to compensate for the decrease in rate. Therefore, the data, when interpreted on a HU basis, indicate that anabolic seed metabolism operates for a longer thermal period in the presence of heat stress, but at greatly reduced biochemical efficiency. Overall, a reduced mature seed size is the result of a decrease in duration without a compensatory increase in rate when expressed on the basis of days or is the result of a decrease in rate without a compensatory increase in duration when expressed on a HU basis. Hence, we recognize the importance of mechanisms that control rate as well as those that control duration, either of which may explain the losses in dry matter accumulation in this study.

Grain Quality

Average kernel density was reduced by heat stress from 1.275 to 1.253 g cm^{-3} ($P = 0.09$), as similarly reported by Lu et al. (1996), but starch, protein, and oil concentrations in the kernel were not changed. This resulted in a decrease in content of starch, protein, and oil ($P = 0.07$, 0.06, and 0.06) similar to reductions in kernel dry weight (Table 4). We are not aware of any other studies that have examined cereal seed starch, protein, and oil constituents simultaneously to examine their responses to heat stress applied specifically during grain filling. Protein and oil concentration does not seem to be affected by August temperature in the U.S. Corn Belt (Earle, 1977). Similarly, high temperatures in a greenhouse study did not affect protein concentration in maize (Lu et al., 1996). In rice, protein percentage in grains declined or was not affected by exposure to high temperature beginning 16 d from heading or later (Tashiro and Wardlaw, 1991).

The parallel decreases in starch, protein, and oil contents caused by chronic heat treatments suggest that losses in kernel dry weight may be produced by one or more mechanisms that broadly affect whole kernel growth. Assimilate transport could be one such mechanism (Wardlaw et al., 1995). The photosynthetic system is another mechanism that may be affected by high temperature during grain filling. For example, Harding et al. (1990) demonstrated that high temperature during reproductive development in wheat seems to affect kernel growth rate and duration by initially accelerating thylakoid component breakdown. Mechanisms of transport and photoassimilation must be considered in studies of heat stress, but perhaps equally important is the effect of high temperature on starch biosynthesis. Numerous studies involving maize (Jones et al., 1984; Keeling et al., 1994; Singletary et al., 1994), wheat (Bhullar and Jenner, 1985), and barley (MacLeod and Duffus, 1988) have shown a negative effect of heat stress on starch deposition in the kernel. A similar series of data showing a negative relationship of protein and oil syntheses to high grain-fill temperature does not exist. We speculate that the negative influence of heat stress on kernel growth in cereals relates to perturbation of starch biosynthesis, but heat stress can also affect the syntheses of other storage products through an indirect effect via interconnected metabolism. It has been reported that coordinated transcriptional regulation between starch and protein syntheses occurs in maize seed (Giroux et al., 1994) and potato (*Solanum tuberosum* L.) tubers (Muller-Rober et al., 1992), which could account for the like response of starch and protein contents to heat stress. Similarly, the ratio of maize endosperm to embryo weight within a specific genotype remains relatively constant regardless of kernel size (Paddick and Sprague, 1939). Because starch is the primary constituent of the endosperm and oil is found mainly in the embryo, it is possible that a drag on starch biosynthesis could concomitantly restrict development of scutellar cells or diminish the deposition of oil in the kernel.

Enzyme Analysis

A survey of enzymes in the pathway of sugar and starch metabolism was performed to determine if the effect of heat stress on kernel growth was associated with altered rates of enzymatic activity. Endosperms were collected (250 postpollination accumulated HU) during the period when kernel growth rate was also being measured and, hence, entailed a stage of temperature-induced growth impairment. Enzyme activity in crude extracts was measured at 25°C for kernels of both temperature treatments. On this basis, high temperature had little effect on most of the enzymes studied, but significantly reduced the endosperm activities of AGPase, glucokinase, sucrose synthase, and SSS (Table 6). Although enzyme activities were measured at 25°C, enzymes in kernels growing at the elevated temperature presumably would have functioned at increased catalytic rates, based upon the temperature coefficient (i.e., Q_{10}). To obtain a better comparison between enzymatic activities in control and heat-stressed kernels, estimates of in vivo activities in the heat-stressed kernels were calculated using Q_{10} values previously determined for the same maize endosperm enzymes according to the same reaction conditions described herein (Table 2; Wilhelm, 1995). Under this premise, activities in developing heat-stressed kernels were equal to or greater than corresponding activities in control kernels in nearly all cases. The only exception was AGPase, where the Q_{10}-adjusted activity was 15% less than control kernels (Table 6).

Despite numerous studies in recent years, determining the aspects of sugar and starch metabolism that are presumably sensitive to conditions of high temperature stress and responsible for the restriction of starch accumulation in heat-stressed seed has remained difficult. Still, it is fair to state that SSS and AGPase are currently given the most credit for restricting starch synthesis under supra-optimal temperatures.

Studies linking specific enzymes with the rate of starch deposition in cereals have suggested SSS as a key control point in the pathway. Catalytically, SSS activity mea-

Table 6. The activity of 11 enzymes of starch and sugar metabolism extracted from heat-treated and control maize endosperm tissue collected at 250 heat units after pollination. Enzyme activity was measured at 25°C for both treatments. For the heat-treated tissue, activites are also reported as a percentage of the control without and after adjustment to the mean of the high temperature treatment (31°C) using the Q_{10} from Table 2.

Enzyme	25/20°C	34/25°C	Heat stress–induced difference	
			Unadjusted	Q_{10} Adjusted
	units per endosperm		% of control	
Sucrose synthase	0.91	0.78	86*	138
UDPglucose pyrophosphorylase	13.3	12.7	95	130
Glucokinase	0.018	0.013	72*	100
ATP-linked fructokinase	0.0065	0.0057	88	182
UTP-linked fructokinase	0.019	0.019	100	132
Phosphoglucoisomerase	1.06	1.05	99	189
Phosphoglucomutase	6.1	5.6	92	133
ATP-linked phosphofructokinase	0.025	0.023	92	112
PPi-linked phosphofructokinase	0.26	0.28	108*	104
ADPglucose pyrophosphorylase	0.20	0.14	70*	85
Soluble starch synthase	0.056	0.049	88*	107

* Significant at $P \leq 0.05$.

sured in vitro becomes less efficient with increasing temperature (Hawker and Jenner, 1993; Jenner et al., 1995), and the enzyme is known to be labile when it, or isolated kernels it is extracted from, are held in vitro at high temperature for short periods (Keeling et al., 1993). Also, the flux control coefficients of other enzymes in the pathway of sugar and starch metabolism in cereal grains, such as branching enzyme, AGPase, and sucrose synthase (Singletary et al., 1997) are much lower than that reported for SSS (Hawker and Jenner, 1993; Jenner et al., 1993; Keeling et al., 1993). Hence, SSS seems to be responsible for the limitation in starch synthesis that occurs under high temperatures. However, in spite of these factors, there are several points that call into question the role of SSS in the temperature sensitivity of starch biosynthesis. Most importantly, simple genetic comparisons of wheat cultivars that show varying degrees of tolerance to heat stress have failed to relate whole-plant differences to catalytic differences in SSS activity measured at high temperatures (Jenner et al., 1993). We have confirmed this lack of correlation using the same cultivars tested by Jenner et al. (1993) and also after comparing SSS and growth data from heat-stressed kernels of several maize inbreds (unpublished results). We also found that starch synthesis in isolated (i.e., in vitro) wheat kernels shows large sensitivities of SSS to temperature (Keeling et al., 1993), but the same was not true for kernels heat treated on the plant (Kuroyanagi and Paulsen, 1988; Hawker and Jenner, 1993). This raises a concern over the agronomic relevance of the in vitro heat treatments, as we have already noted. It is noteworthy that the activity profile of SSS is compressed in time and amount very similarly to AGPase, and the in vitro measured activity of SSS is low relative to most enzymes of sugar and starch biosynthesis (Caley et al., 1990; Singletary et al., 1994). In our study, Q_{10}-corrected SSS activity was slightly increased, which may directly contribute to the small increase in grain-fill rate. Similarly, heat induces a gradual decline in SSS activity, which can lead to a premature end to starch biosynthesis (Singletary et al., 1994).

The response of AGPase is, in some ways, the opposite of SSS in response to high temperatures. In vitro, AGPase activity is stable at high temperature (Kennedy and Isherwood, 1975; Keeling et al., 1993) and its activity profile during development of maize under heat stress is more reduced in magnitude and timing than that of SSS (Ou-Lee and Setter, 1985; Singletary et al., 1994). Work by Duke and Doehlert (1996) involving heat-stressed kernels of maize also demonstrates that AGPase activity is constricted more than several other enzymes of sugar metabolism. In addition, only 5 d of growth at 30°C, compared with 25°C, reduced the expression of AGPase genes Brittle-2 and Shrunken-2 by 50 and 70%, respectively. On the other hand, simple genetic comparisons of wheat cultivars known to vary in heat-stress tolerance failed to relate AGPase activity to whole-plant differences, similar to SSS (Jenner et al., 1993). In our study of 11 enzymes, Q_{10}-adjusted AGPase activity was the most reduced by high temperature. This suggests that high temperature affects AGPase more than the other enzymes, which may contribute to a reduction in the efficiency of starch and dry matter accumulation or cause a premature termination of grain-filling (Singletary et al., 1994). However, previous studies indicate that reductions in starch accumulation generally do not occur without a decrease in AGPase activity of 40% or more (Singletary et al., 1994, 1997). This calls into question the importance of a 15% reduction in AGPase activity in reducing final seed size, as in our study.

The roles of glucokinase and sucrose synthase in determining starch accumulation in heat-treated tissues are less clear. In our study, high temperature had an adverse effect on the activity of both of these enzymes before Q_{10}-adjustment. Previous studies have shown high-temperature sensitivity of glucokinase in maize endosperm tissue (Singletary et al., 1994) and sucrose synthase in both maize (Singletary et al., 1994) and barley (MacLeod and Duffus, 1988), and reductions in activity of these enzymes may be linked to a reduction in starch biosynthesis.

In conclusion, the seed growth response to heat stress was not significantly different across seven maize genotypes. A heat stress of moderate magnitude, but of much greater duration than normally occurs in the U.S. Corn

Belt, reduced kernel dry weight by a small but significant amount (7%). Reductions in kernel dry weight were attributable to proportional losses in starch, protein, and oil contents, indicating that a mechanism affecting whole grain metabolism is responsible for the loss. Grain-filling duration in HU increased under heat stress, but could not compensate for the decrease in grain-filling rate, producing kernel dry weight losses. The chronic high temperature significantly reduced the activities of four enzymes of the starch biosynthetic pathway: AGPase, SSS, glucokinase, and sucrose synthase, but only AGPase activity was reduced when activities were Q_{10}-corrected to the growth temperature. These results indicate that these enzymes are closely linked to reductions in starch content and may explain the reductions in mature seed dry weight.

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Influence of Nitrogen Availability on Seed Nitrogen Accumulation in Pea

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ABSTRACT

The final seed nitrogen concentration (the ratio of seed N and dry matter content) is highly variable in pea (*Pisum sativum* L.) and N remobilization during seed filling may limit yield by restricting the seed filling period. This study was conducted to determine how seed N accumulation is regulated in pea. The effect of N availability and distribution on individual seed N accumulation rate at different nodes was investigated in three genotypes grown in the field and glasshouse under various levels of N fertilizer, depodding, and defoliation. The N content of vegetative plants parts (stems, leaves, podwalls) and seeds from three mainstem nodes were regularly recorded. Plant N available to the seeds at a given time was assessed as the sum of the amount of N still available for remobilization in vegetative parts and the amount of N accumulated by the plant. The results indicate that the N available in a plant at a given time can be considered as one common pool accessible to all seeds and equitably divided among them. Thus, the rate of individual seed N accumulation was unaffected by intra-plant position of seeds. This rate increased with the amount of N available per seed until a maximum rate of individual seed N accumulation (43 μg seed^{-1} degree-day^{-1}) was reached. The relationship established between the rate of individual seed N accumulation and the plant N available per seed will be useful to improve models simulating yield and final seed N concentration in legumes.

THE SEED NITROGEN CONCENTRATION of pea varies greatly with environment (Karjalainen and Kortet, 1987). Several studies on different species have shown that changes in assimilate availability during the filling period lead to changes in the seed N concentration. Nitrogen stresses during seed filling caused a decrease in seed N concentration (Streeter, 1978 with soybean [*Glycine max* (L.) Merrill]; Holl and Vose, 1980 with pea); enhancing assimilate availability after the beginning of the filling period by seed removal led to an increase in seed N concentration [Jones and Simmons, 1983 with maize (*Zea mays* L.)]. This variability in seed N concentration may play an important role in the determination of seed yield because legume plants are characterized by massive nitrogen remobilization from vegetative parts to provide N to the growing seed (Sinclair and de Wit, 1975, 1976; Pate, 1985). Since the loss of N from vegetative parts causes a decrease in plant photosynthetic capacity (Sinclair and Horie, 1989), the plant senesces and the premature termination of seed filling can limit yield.

At each nodal position, the changes in seed N concentration during seed filling can be analyzed as the ratio of the seed N accumulation rate and the seed growth rate. Changes in the assimilate availability at the time when seed number cannot be altered have no effect on seed growth rate [Jones and Simmons, 1983 with maize; Munier-Jolain et al., 1998 with pea, lupin (*Lupinus albus* L.), and soybean]. The seed growth rate is related to the cotyledon cell number which is fixed before the beginning of seed filling (Egli et al., 1989; Guldan and Brun, 1985 with soybean; Munier-Jolain and Ney, 1998 with pea and soybean). In contrast, N accumulation of in vitro cultured seed was positively correlated with the medium N concentration (soybean: Hayati et al., 1996; maize: Singletary and Below, 1989). Thus, the variations in seed N concentration during the filling period could be mainly due to changes in the seed N accumulation rate which seems to depend on N assimilate availability.

Pea is an indeterminate plant whose pods and seeds are set on successive reproductive nodes at different heights of the plant. The development of pods from various positions is asynchronous and the seeds from higher pods begin to fill later than seeds from lower pods (Ney and Turc, 1993). This characteristic may lead to variations in the seed N accumulation rate between nodes. As N concentration in vegetative parts increases

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Abbreviations: BSL, beginning of seed filling at the last reproductive node; DW, dry weight; NA, amount of nitrogen available to seeds; NAS, nitrogen available per seed; Nremob; amount of remobilizable nitrogen; Nveg, nitrogen content in vegetative parts; SNR, individual seed nitrogen accumulation rate.